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Purpose and scope: We are conducting a large case-control study nested within a prospective cohort, to estimate relative risks of breast cancer by serum levels of IGF-I and IGFBP-3, and to examine associations of polymorphisms in 15 candidate genes with levels of IGF-I, IGFBP-3, and cancer risk.
Progress report: In the first two years of this project, 1084 breast cancer cases and 2116 control subjects were included in the study, and measurements of IGF-I and IGFBP-3 were (practically) completed. A first series of 23 SNPs were typed for all case and control subjects, and typing of 18 additional SNPs is ongoing.
Major findings: Elevated serum IGF-I shows a moderate increase in breast cancer risk among postmenopausal women. Preliminary analyses also show significant associations of a number of SNPs and haplotypes with IGF-I, IGFBP-3 or breast cancer risk.

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INTRODUCTION

IGF-I is a central hormone in the regulation of anabolic (growth) processes as a function of available energy and elementary substrates (e.g., essential amino acids), and has strongly mitogenic and anti-apoptotic activities. Results from *in vitro* studies and animal experiments show that, in excess, the anabolic signals by IGF-I can promote the development of tumors at various organ sites, and recent epidemiological studies have shown an increased breast cancer risk in women with elevated serum IGF-I, or with elevated levels of IGF-I for given levels of IGFBP-3, the major plasmatic IGF-binding protein.

While nutritional status is one important determinant of circulating IGF-I levels, heritability studies have shown that, in well-nourished populations, a large part (40-60 %) of variation in IGF-I is (co) determined by genetic factors. To increase understanding of what are the major determinants of IGF-I levels, as well as cancer risk, we have conducted a study with the following objectives:

1. Confirm that elevated prediagnostic serum levels of IGF-I increase breast cancer risk, especially in premenopausal women;
2. Describe exhaustively existing polymorphisms, allele frequencies and haplotypes in 13 selected genes related to the secretion of growth hormone, and hence to the synthesis of IGF-I and IGFBP-3; and
3. Examine whether these genetic polymorphisms are related to significant increases or decreases in circulating levels of IGF-I and IGFBP-3, as well as in breast cancer risk.

Our project is a large case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC), using prediagnostic blood (serum and DNA) samples collected during 1992-1998, from 233,800 women in Western Europe.

BODY

Our original grant application stated the following specific aims:

1. Conduct a case-control study (about 1,000 breast cancer cases and 1,000 matched control subjects) nested within the prospective EPIC cohorts, to estimate the associations of serum IGF-I and IGFBP-3 levels with breast cancer risk;
2. Preparation of an exhaustive catalogue of polymorphisms and haplotypes in 13 selected candidate genes (Table 1), and a ("phase-1") association study on a subset of 400 control subjects to identify genotypes that have a minimum level of association with serum of IGF-I and IGFBP-3; selection of a subset of SNPs that would be worth genotyping in the full study of breast cancer cases and controls;
3. Conduct a nested case-control study ("phase-2"), to estimate relative risks of breast cancer in relation to the genotypes of SNPs selected in phase-1;
4. Parallel analysis ("phase-2") of associations of these selected genotypes with serum levels of IGF-I and IGFBP-3, in all breast cancer cases and control subjects.

Table 1. Selected candidate genes for study of associations with circulating IGF-I and IGFBP-3, and with breast cancer risk.

Gene	Name and Function of gene product
IGF-I	Insulin-like growth factor-I
IGFBP-3	IGF-binding protein-3: major plasmatic binding protein of IGF-I
GH1	Growth hormone: Main stimulus for synthesis of IGF-I and IGFBP-3
GHR	Growth hormone receptor: mediates GH effects
GHRH	Growth hormone releasing hormone: stimulates pituitary GH release
GHRHR	Growth hormone releasing hormone receptor; Mediates GHRH effects
SST	Somatostatin; inhibits pituitary GH release
SSTR1 – SSTR5	Somatostatin receptors, types 1 - 5; mediate SST effects on pituitary GH release
POU1F1	Pituitary-specific transcription factor; crucial for pituitary GH synthesis

All of the above aims have been fully met, and have even been extended by the inclusion of additional candidate genes in the same metabolic pathway (*IGFALS*, *GHRL*, *GHSR*, and *IGF1R*). We have not yet entirely finished the preparation and submission of reports for publication in scientific, specialized journals; nevertheless, five articles have been prepared, 2 of which have been submitted, 3 are in draft format and a further two papers are planned to be completed within the coming six months.

KEY RESEARCH ACCOMPLISHMENTS

Summary of progress reports years 1 and 2

Over the first two years of our project, progress in our work has been regular and can be summarized as follows:

Year 1:

Case-control selection, DNA extraction, and growth factor measurements:

- An almost complete selection of breast cancer cases (n = 810) and control subjects (n = 1620) within the EPIC cohorts, for the nested case-control study on serum IGF-I, IGFBP-3 and breast cancer risk;
- Measurement of IGF-I and IGFBP-3 for these cases and controls;
- DNA extraction for these cases and controls;

Identification and selection of SNPs, to be typed in the full case-control study:

- Identification of a comprehensive catalogue of over 120 single nucleotide polymorphisms (SNPs) in all candidate genes included in the present study;
- Preparation of a DNA genotyping chip, for 78 of these polymorphisms that had confirmation of being true polymorphisms;
- Conduct of a first descriptive study of SNP and haplotype frequencies for all candidate genes, in a population of 477 subjects, and a preliminary analysis of associations of these SNPs with serum levels of IGF-I and IGFBP-3; selection of SNPs to be typed in the full case-control study;
- genotyping of a first series of 23 SNPs in the full case-control study, using Taqman assays.

Year 2:

- Inclusion in the study of 274 additional breast cancer cases and 496 additional control subjects, bringing the total numbers of cases and controls to 1,084 and 2,116, respectively;
- DNA extraction for these additional study subjects
- Measurement of IGF-I and IGFBP-3 for these additional study subjects;
- Genotyping of a first series of 23 SNPs, for all cases and controls, thus bringing the total of SNPs typed to 41, for all 1084 breast cancer cases and 2116 control subjects; and
- Provisional statistical analyses of relationships between genetic polymorphisms, circulating IGF-I and IGFBP-3, and breast cancer risk.

Details of our progress during years 1 and 2 are in our previous (progress) reports.

Year 3:

During the third, and final year of our project, key accomplishments were the following:

- Completion of genotyping of 18 additional SNPs identified in the later phase of the study (mostly from public databases and recent literature);
- Definitive statistical analyses of the complete SNP genotype and haplotype data, in relation to serum IGF-I and IGFBP-3, as well as to breast cancer risk;
- Definitive statistical analysis of relationships of breast cancer risk in relation to serum levels of IGF-I and IGFBP-3.
- Preparation of articles, to report all major findings in scientific journals.

Summary of main study results

Contrary to several previous reports, our study showed no relationship of serum IGF-I and/or IGFBP-3 with risk of breast cancer diagnosed at an approximately premenopausal age. Among postmenopausal women, however, there was a moderate association of risk with both IGF-I and IGFBP-3 (relative risks of about 1.5 between the extreme quintiles of the IGF-I exposure distribution). A paper reporting these findings has been prepared, and will soon be submitted to a scientific journal (1).

Besides associations of IGF-I and IGFBP-3 with breast cancer risk, we have also performed two spin-off studies to examine relationships of these peptides with body mass index, and with dietary intakes of major food groups and nutrients. These results were reported in two articles that have been both submitted for publication (2-3).

With respect to genetic polymorphism data, final statistical analyses were performed on a total of 807 cases of breast cancer, and 1588 matched control subjects. These numbers of cases and controls are lower than those (1084 breast cancer cases and 2116 control subjects) for whom genotyping of SNPs had been completed. The reason for this difference is that we have preferred to exclude part of the data, because of incomplete or unbalanced matching of case- and control subjects for some of the EPIC recruitment centers. This incomplete matching could not be avoided, because of the ways DNA extraction plates had been prepared upon initial DNA extraction from buffy coats (partially, this had been started before our project), and our lack of capacity to efficiently rearrange these plates with our existing laboratory equipment. In the coming years our study will be extended, however, as part of a collaborative project with an international Cohort Consortium (including 6 additional cohorts in the USA and Europe), and this will eventually allow us to include also those breast cancer cases and control subjects that, for the purpose of our current analyses and publications, for the moment have been left out of our statistical analyses.

Statistical analyses on the polymorphism data were performed with a variety of statistical models, of increasing completeness and complexity:

- *At the level of individual SNP loci*: Associations of alleles with IGF-I and IGFBP-3 concentrations were estimated under assumption of dominance, codominance, or recessiveness of alleles.
- *At the level of full gene loci*: Individuals' most likely haplotype dosages (i.e., posterior probability of each of the possible haplotypes given an individual's SNP genotypes) were estimated using a method and computer programme by Stram et al. (4), and were related to breast cancer risk and serum levels of IGF-I and IGFBP-3. Each of these models were also performed under assumption of either a codominant, dominant, or recessive inheritance.

These analyses resulted in a rather large number of results, and showed numerous associations, with some degree of statistical significance, with regard to serum IGF-I, IGFBP-3, or breast cancer risk (Tables 2-6)

Major findings were the following:

- SST and SSTR2 polymorphisms showed weak but statistically significant associations with breast cancer risk. SSTR5 polymorphisms were associated with IGF-I levels, while one polymorphism in GHRHR and one in POU1F1 were associated with IGFBP-3 levels. However, none of the polymorphisms were associated at the same time with cancer risk and hormone levels, which suggests that the observed associations with cancer risk are not mediated by alterations in circulating IGF-I or IGFBP-3 levels.
- There was a weak but nominally significant association of a block of polymorphisms located at the 5' of the *IGF1* gene with breast cancer risk, particularly among women younger than 55, and a strong association of polymorphisms located in the 5' region of *IGFBP3* with circulating levels of IGFBP-3, which confirms observations from previous studies.

In none of the other genes did polymorphisms show any major associations with breast cancer risk, although some showed marginal associations with circulating IGF-I or IGFBP-3.

REPORTABLE OUTCOMES

Five articles have been prepared, two of which are submitted. Two major papers (5-6) to report the key findings relating SNP genotypes and haplotypes to IGF-I, IGFBP-3 and breast cancer risk will be submitted in the near future.

- Sabina Rinaldi, Laure Dossus, Carine Biessy, Franco Berrino, Tim Key, Pilar Amiano, Sheila Bingham, Heiner Boeing, H. Bas Bueno de Mesquita, Jenny Chang-Claude, Françoise Clavel-Chapelon, Agnès Fournier, Carla H van Gils, Carlos A Gonzalez, Aurelio Barricarte Gurrea, E. Kritseli, Kay Tee Khaw, Vittorio Krogh, Petra Lahmann, Gabriele Nagel, Anja Olsen, Charlotte Onland-Moret, Kim Overvad, Domenico Palli, Petra Peeters, Jose R Quiros, Andrew Roddam, Anne Thiebaut, Anne Tjønneland, Maria-José Tormo, Antonia Trichopoulou, Dimitrios Trichopoulos, Rosario Tumino, Paolo Vineis, Teresa Norat, Pietro Ferrari, Nadia Slimani, Elio Riboli, Rudolf Kaaks. IGF-I, IGFBP-3 AND breast cancer risk in women: the European Prospective Investigation into cancer and nutrition (EPIC). **DRAFT**

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- Federico Canzian, James McKay, Rebecca Cleveland, Laure Dossus, Carine Biessy, Catherine Boillot, Sabina Rinaldi, *authors from other EPIC centers to be added: two other IARC authors to be added*, Elio Riboli, Rudolf Kaaks. Genetic variation in the growth hormone synthesis pathway in relation to circulating IGF-I, IGFBP-3, and breast cancer risk: results from the EPIC study. **CURRENTLY CIRCULATING AMONG EPIC COLLABORATORS FOR COMMENTS, AND TO BE SUBMITTED FOR PUBLICATION SOON.**

- Federico Canzian, James McKay, Rebecca Cleveland, Laure Dossus, Carine Biessy, Catherine Boillot, Sabina Rinaldi, Stephanie Monnier, *authors from other EPIC centers to be added, two other IARC authors to be added*, Elio Riboli, Rudolf Kaaks. Polymorphisms of genes coding for insulin-like growth factor-I and its major binding proteins, circulating levels of IGF-I and IGFBP-3 and breast cancer risk: results from the EPIC study. **CURRENTLY CIRCULATING AMONG EPIC COLLABORATORS FOR COMMENTS, AND TO BE SUBMITTED FOR PUBLICATION SOON.**

Two more papers are being planned, on the following topics:

- Polymorphic variation in the *GHRL* and *GHSR* genes, in relation to circulating IGF-I, IGFBP-3, and breast cancer risk, in the EPIC cohort; **IN PREPARATION, TO BE FINALIZED BEFORE DECEMBER 2004;** and

- A polygenic prediction model of circulating IGF-I and IGFBP-3 levels. **PLANNED DATE OF FINALIZATION: APRIL 2005**

Overview of results from statistical analyses on genetic polymorphisms.

Table 2. Association between individual SNPs and breast cancer risk

GENE	SNPs	ALL WOMEN	LESS THAN 55	MORE THAN 55
SST	P0692	Hetero: 1.31 (1.06-1.61) P_{cod}=0.04; P_{dom}=0.02	NS	Hetero: 1.53 (1.17-2.01) P_{cod}=0.01; P_{dom}=0.003
	P0689	Hetero: 1.27 (1.02-1.59) P_{cod}=0.04; P_{dom}=0.03	NS	Hetero: 1.41 (1.07-1.88) P_{cod}=0.03; P_{dom}=0.02
SSTR1	P0694	NS	NS	Hetero: 0.76 (0.59-0.99)
SSTR2	P0837	NS	Hetero: 1.39 (1.03-1.38)	NS
	P0836	Homo: 0.74 (0.57-0.96) P_{cod}=0.04; P_{rec}=0.02	NS	NS
SSTR3	P0702	NS	NS	NS
SSTR4	P0710	NS	NS	NS
SSTR5	P0719	NS	NS	NS
	P0723	NS	NS	NS
	P0727	NS	NS	NS
	P0827	NS	NS	NS
GH1	P0320	NS	Hetero: 0.71 (0.52-0.96) P_{dom}=0.03	NS
	P0327	NS	NS	NS
	P0323	Hetero: 0.77 (0.63-0.94) P_{dom}=0.03	NS	NS
	P0322	NS	NS	NS
GHR	P0329	NS	NS	P_{rec}=0.05
	P0332	NS	NS	NS
	P0335	NS	NS	NS
GHRH	P0342	NS	NS	NS
GHRHR	P0347	NS	NS	NS
	P0348	NS	NS	NS
	P0353	NS	NS	NS
	P0359	NS	NS	NS
	P0360	NS	NS	NS
POU1F1	P0593	NS	NS	NS
GHRELIN	P0341	NS	NS	Hetero: 1.32 (1.00-1.75)
	P0340	Hetero: 1.29 (1.00-1.68) P_{cod}=0.04; P_{dom}=0.04	P_{cod}=0.05	NS
	P0337	NS	NS	NS
	P0338	NS	NS	NS
GHSR	P0831	Homo: 2.46 (1.18-5.13) P_{rec}=0.02	NS	NS
	P0363	NS	Hetero: 0.72 (0.53-0.98) Homo: 1.71 (1.03-2.82) P_{rec}=0.01	NS
	P0366	NS	NS	NS

Table 2. (continued)

GENE	SNPs	ALL WOMEN	LESS THAN 55	MORE THAN 55
IGFBP3	P0846	NS	NS	NS
	P0448	NS	NS	NS
	P0455	NS	NS	Hetero: 0.74 (0.57-0.96) P_{dom}=0.03
	P0453	NS	NS	NS
	P0450	NS	NS	NS
	P0832	NS	NS	NS
	P0844	NS	NS	NS
	P0845	Hetero: 0.83 (0.69-0.99)	NS	NS
IGF1	P0429	NS	NS	NS
	P0425	NS	NS	NS
	P0416	Homo: 0.57 (0.34-0.97) P_{cod}=0.03; P_{rec}=0.05	Homo: 0.17 (0.07-0.56) P_{cod}=0.002; P_{dom}=0.01; P_{rec}=0.005	NS
	P0419	P_{cod}=0.04	Homo: 0.39 (0.12-0.83) P_{cod}=0.005; P_{dom}=0.01; P_{rec}=0.03	NS
	P0415	NS	P_{cod}=0.03; P_{dom}=0.05	NS
IGF1R	P0437	NS	NS	NS
	P0847	NS	NS	NS
	P0848	NS	NS	NS
IGF1ALS	P0829	NS	NS	NS
	P0439	NS	NS	P_{cod}=0.02; P_{dom}=0.04
	P0828	NS	NS	NS

Table 3. Associations between individual SNPs and breast cancer risk by age at diagnosis.

GENE	SNPs	ALL WOMEN	LESS THAN 55	MORE THAN 55
SST	P0692	Hetero: 1.31 (1.06-1.61) P_{cod}=0.04; P_{dom}=0.02	NS	Hetero: 1.53 (1.17-2.01) P_{cod}=0.01; P_{dom}=0.003
	P0689	Hetero: 1.27 (1.02-1.59) P_{cod}=0.04; P_{dom}=0.03	NS	Hetero: 1.41 (1.07-1.88) P_{cod}=0.03; P_{dom}=0.02
SSTR1	P0694	NS	NS	Hetero: 0.76 (0.59-0.99)
SSTR2	P0837	NS	Hetero: 1.39 (1.03-1.38)	NS
	P0836	Homo: 0.74 (0.57-0.96) P_{cod}=0.04; P_{rec}=0.02	NS	NS
SSTR3	P0702	NS	NS	NS
SSTR4	P0710	NS	NS	NS
SSTR5	P0719	NS	NS	NS
	P0723	NS	NS	NS
	P0727	NS	NS	NS
	P0827	NS	NS	NS
GH1	P0320	NS	Hetero: 0.71 (0.52-0.96) P_{dom}=0.03	NS
	P0327	NS	NS	NS
	P0323	Hetero: 0.77 (0.63-0.94) P_{dom}=0.03	NS	NS
	P0322	NS	NS	NS
GHR	P0329	NS	NS	P_{rec}=0.05
	P0332	NS	NS	NS
	P0335	NS	NS	NS
GHRH	P0342	NS	NS	NS
GHRHR	P0347	NS	NS	NS
	P0348	NS	NS	NS
	P0353	NS	NS	NS
	P0359	NS	NS	NS
	P0360	NS	NS	NS
POU1F1	P0593	NS	NS	NS
GHRELIN	P0341	NS	NS	Hetero: 1.32 (1.00-1.75)
	P0340	Hetero: 1.29 (1.00-1.68) P_{cod}=0.04; P_{dom}=0.04	P_{cod}=0.05	NS
	P0337	NS	NS	NS
	P0338	NS	NS	NS
GHSR	P0831	Homo: 2.46 (1.18-5.13) P_{rec}=0.02	NS	NS
	P0363	NS	Hetero: 0.72 (0.53-0.98) Homo: 1.71 (1.03-2.82) P_{rec}=0.01	NS
	P0366	NS	NS	NS

Table 3. (continued)

GENE	SNPs	ALL WOMEN	LESS THAN 55	MORE THAN 55
IGFBP3	P0846	NS	NS	NS
	P0448	NS	NS	↓ IGF1: P_{cod}=0.02; P_{dom}=0.03
	P0455	NS	NS	NS
	P0453	↑IGF1: P_{cod}=0.001; P_{dom}=0.001	↑IGF1: P_{cod}=0.02; P_{dom}=0.02	NS
	P0450	↓ IGFBP3: P_{cod}<0.0001; P_{dom}=0.0001; P_{rec}=0.001	↓ IGFBP3: P_{cod}=0.01; P_{dom}=0.03	↓ IGFBP3: P_{cod}=0.0002; P_{dom}=0.002; P_{rec}=0.001
	P0832	↑ IGFBP3: P_{cod}<0.0001; P_{dom}<0.0001; P_{rec}<0.0001	↓ IGF1: P_{dom}=0.03 ↑ IGFBP3: P_{cod}=0.0001; P_{dom}=0.0001; P_{rec}=0.01	↑ IGFBP3: P_{cod}<0.0001; P_{dom}<0.0001; P_{rec}<0.0001
	P0844	↑IGF1:P_{rec}=0.002 ↓ IGFBP3: P_{cod}=0.01; P_{dom}=0.003	↑IGF1: P_{rec}=0.01	↑IGF1: P_{rec}=0.04 ↓ IGFBP3: P_{cod}=0.04; P_{dom}=0.02
	P0845	↓ IGFBP3: P_{cod}<0.0001; P_{dom}<0.0001; P_{rec}<0.0001	↓ IGFBP3: P_{cod}=0.0004; P_{dom}=0.005; P_{rec}=0.002	↓ IGFBP3: P_{cod}<0.0001; P_{dom}<0.0001; P_{rec}=0.0003
IGF1	P0429	NS	NS	↓ IGFBP3: P_{cod}=0.03; P_{dom}=0.02
	P0425	NS	NS	NS
	P0416	↓ IGFBP3: P_{rec}=0.003	NS	↓ IGFBP3: P_{rec}=0.05
	P0419	↑ IGF1: P_{dom}=0.02 ↓ IGFBP3: P_{rec}=0.02	NS	NS
	P0415	↑ IGF1: P_{dom}=0.02	NS	NS
IGF1R	P0437	NS	NS	↓ IGF1: P_{dom}=0.03
	P0847	↑ IGFBP3: P_{rec}=0.02	↑ IGFBP3: P_{rec}=0.01	NS
	P0848	NS	NS	NS
IGF1ALS	P0829	NS	NS	NS
	P0439	NS	NS	NS
	P0828	↓ IGF1: P_{cod}=0.02; P_{rec}=0.001 ↓ IGFBP3: P_{cod}=0.03; P_{rec}=0.05	↓ IGF1: P_{cod}=0.04; P_{rec}=0.02	NS

Table 4. Associations of genetic polymorphisms (SNPs) with IGF-I or IGFBP-3, by age at diagnosis.

GENE	SNPs	ALL WOMEN	LESS THAN 55	MORE THAN 55
SST	P0692	NS	NS	↑ IGFBP3: P_{cod}=0.03; P_{dom}=0.03
	P0689	NS	NS	↑ IGFBP3: P_{cod}=0.01; P_{dom}=0.02
SSTR1	P0694	NS	NS	NS
SSTR2	P0837	NS	NS	NS
	P0836	NS	NS	NS
SSTR3	P0702	NS	↑ IGFBP3: P_{dom}=0.03	NS
SSTR4	P0710	NS	NS	NS
SSTR5	P0719	NS	NS	NS
	P0723	NS	NS	NS
	P0727	NS	NS	NS
	P0827	↑ IGF1 P_{cod}=0.01; P_{dom}=0.01	↑ IGF1 P_{cod}=0.01; P_{dom}=0.004	↑ IGFBP3: P_{rec}=0.05
GH1	P0320	NS	NS	NS
	P0327	NS	NS	NS
	P0323	NS	NS	NS
	P0322	NS	NS	NS
GHR	P0329	NS	NS	NS
	P0332	NS	NS	NS
	P0335	NS	NS	NS
GHRH	P0342	NS	NS	NS
GHRHR	P0347	NS	NS	NS
	P0348	NS	NS	NS
	P0353	↑ IGFBP3: P_{rec}=0.03	NS	NS
	P0359	NS	↓ IGFBP3: P_{rec}=0.04	NS
	P0360	NS	NS	NS
POU1F1	P0593	↓ IGFBP3: P_{cod}=0.004; P_{dom}=0.006	↑ IGF1: P_{dom}=0.05	↓ IGFBP3: P_{cod}=0.003; P_{dom}=0.01; P_{rec}=0.03
GHRELIN	P0341	↓ IGFBP3: P_{cod}=0.01; P_{rec}=0.02	NS	↓ IGFBP3: P_{cod}=0.01; P_{rec}=0.03
	P0340	NS	↑ IGF1: P_{rec}=0.04	NS
	P0337	NS	NS	NS
	P0338	NS	NS	NS
GHSR	P0831	NS	↑ IGFBP3: P_{rec}=0.03	NS
	P0363	NS	NS	NS
	P0366	NS	NS	NS

Table 5. Association between haplotypes and breast cancer risk

GENE	ALL WOMEN	LESS THAN 55	MORE THAN 55
SST	hCC: cod:1.24 (1.01-1.52); dom:1.27 (1.02-1.59)	NS	NS
SSTR2	hGG : dom: 1.24 (1.03-1.51)	hGG : dom: 1.36 (1.01-1.83)	NS
SSTR5	NS	NS	NS
GH1	NS	NS	hTCAG: dom: 1.38 (1.04-1.82)
GHR	NS	NS	NS
GHRHR	NS	NS	hCGTTC: dom: 1.43 (1.01-2.02)
GHRELIN	NS	hGTAT: cod: 1.74 (1.10-2.75); dom: 1.63 (1.02-2.60) hTAAT: cod: 1.69 (1.06-2.70)	NS
GHSR	hGGC : rec: 2.50 (1.20-5.20)	hAAT : rec: 1.95 (1.21-3.13)	NS
IGFBP3	hATACGGGA: cod: 0.52 (0.29-0.95); dom:0.51 (0.27-0.94)	hTTACCGGA: rec:4.69 (1.14-19.23)	hATACGGGA: cod:0.46 (0.22-0.95); dom: 0.45 (0.21-0.95)
IGF1	hTTGTC: cod; 0.40 (0.20-0.81); dom: 0.40 (0.20-0.81)	NS	hTTGTC: cod: 0.29 (0.09-0.89); dom: 0.27 (0.09-0.86)
IGF1R	NS	NS	NS
IGF1ALS	NS	NS	hGGT: cod:1.34 (1.01-1.76)

Table 6. Association between haplotypes and IGF-I and IGFBP-3 levels adjusted for age and center

GENE	ALL WOMEN	LESS THAN 55	MORE THAN 55
SST	NS	NS	hCC: ↑ IGFBP3 (cod: 118.26 (0.01); dom: 120.63 (0.02))
SSTR2	NS	NS	NS
SSTR5	hCCCA: ↑ IGF1 (cod: 16.32 (0.01); dom: 17.05 (0.01))	hCCCA: ↑ IGF1 (cod: 25.84 (0.01); dom: 29.96 (0.003))	hCCCA: : ↑ IGFBP3 (rec: 1466.11 (0.05))
GH1	hTCAT: ↓ IGF1 (cod: -5.43 (0.04); dom: -6.82 (0.03)) hTCGG: ↓ IGF1 (cod: -8.16 (0.05); dom: -46.80 (0.01))	NS	hACAG: ↓ IGF1 (rec: -80.82 (0.01)) hTCGG : ↓ IGF1 (rec: -60.90 (0.01)); ↓ IGFBP3 (rec: -551.96 (0.02))
GHR	NS	hGGT: ↓ IGFBP3 (rec: -227.61 (0.01))	NS
GHRHR	hCGCCG: ↑ IGFBP3 (rec: 121.82 (0.05))	hCGCCC: ↓ IGF1 (cod: -38.99 (0.01); dom: -38.20 (0.02)) hCGTTC: ↓ IGF1 (cod: -12.15 (0.04)); ↓ IGFBP3 (rec: -349.67 (0.03))	NS
GHRELIN	hGACT: ↑ IGFBP3 (rec: 182.66 (0.01)) hTAAT: ↓ IGF1 (rec: -45.29 (0.04)) hTACT: ↓ IGF1 (cod: -10.74 (0.04)	hTAAT: ↓ IGFBP3 (dom: -145.19 (0.04)) hGACT: ↑ IGFBP3 (rec: 260.16 (0.002))	hTACT: ↓ IGF1 (cod: -13.79 (0.03); dom: -14.20 (0.04))
GHSR	NS	hGGC: ↑ IGFBP3 (rec: 438.34 (0.04))	hAGC: ↓ IGF1 (cod: -8.08 (0.02); rec: -20.60 (0.03))

Table 6. (continued)

GENE	ALL WOMEN	LESS THAN 55	MORE THAN 55
IGFBP3	▪ hATACGTGG: ↑ IGF1 (rec: 18.74 (0.02)); ↑ IGFBP3 (cod: 136.49 (0.0001); dom: 122.82 (0.002)) ▪ hACACGTGG: ↑ IGFBP3 (cod: 107.69 (0.007); dom: 95.50 (0.02)) ▪ hTTACCGGA: ↓ IGFBP3 (dom: -103.64 (0.02)) ▪ hTTACGTGG: ↑ IGFBP3 (cod: 159.65 (0.0004); dom: 146.59 (0.002)) ▪ hATGCGTGG: ↑ IGFBP3 (cod: 104.84 (0.04))	▪ hATGCGGGA: ↓ IGF1 (cod: -14.28 (0.05); rec: (-53.20 (0.05)) ▪ hTTACGTGG: ↓ IGF1 (cod: -15.62 (0.03)) ▪ hTTACCGGA: ↑ IGF1 (rec: 44.63 (0.05))	▪ hTTACCGGA: ↓ IGF1 (dom: -14.29 (0.02)); ↓ IGFBP3 (cod: -139.60 (0.03); dom: -192.97 (0.004)) ▪ hATACGTGG: ↑ IGF1 (rec: 24.68 (0.01)); ↑ IGFBP3 (cod: 146.74 (0.003); dom: 120.75 (0.03); rec: 305.73 (0.004)) ▪ hACACGTGG: ↑ IGFBP3 (cod: 112.48 (0.04)) ▪ hTTACGTGG: ↑ IGFBP3 (cod: 224.48 (0.0004); dom: 213.04 (0.001)) ▪ hATACGGGA: ↓ IGFBP3 (rec: -867.81 (0.04))
IGF1	▪ hCCACC: ↑ IGF1 (dom: 9.65 (0.05) ▪ hCCGTA: ↑ IGF1 (cod: 9.25 (0.04); dom: 12.56 (0.01)) ▪ hTCGTC: ↓ IGFBP3 (cod: -179.43 (0.03); dom (-181.79 (0.02)) ▪ hTTGTC: ↓ IGFBP3 (cod: -285.84 (0.003); dom (-250.87 (0.01))	▪ hCTGTA: ↑ IGF1 (cod: 43.29 (0.04); dom: 41.43 (0.05))	▪ hCCGTA: ↑ IGF1 (dom:12.94 (0.04)) ▪ hTCACC: ↓ IGF1 (rec:-38.87 (0.04)) ▪ hTTACC: ↓ IGF1 (rec: -15.49 (0.05)) ▪ hTCGTC: ↓ IGFBP3 (cod: -218.98 (0.05); dom: -219.17 (0.05)) ▪ hTTACC: ↓ IGFBP3 (cod: -80.30 (0.05); rec: -177.83 (0.04)) ▪ TTGTC: ↓ IGFBP3 (cod: -415.07 (0.005); dom: -336.07 (0.03); rec: -2672.82 (0.0002))
IGF1R	NS	NS	NS
IGF1ALS	hGCC: ↓ IGF1 (cod: -6.21 (0.02); rec: -27.93 (0.001)) ↓ IGFBP3 (cod: -59.79 (0.03); rec: -173.22 (0.04))	hGCC: ↓ IGF1 (cod: --8.54 (0.04); rec: -28.16 (0.02))	NS

Cod = codominant
Dom= dominant
Rec= recessive

CONCLUSIONS

We have met all major study objectives, and actually even exceeded these in terms of the total number of SNPs genotyped and candidate genes examined: Four candidate genes were added, and the total number of SNPs typed was 41, which is a considerably larger number of SNPs than the number (15 SNPs) that we had originally anticipated when writing our study proposal. In addition, the total study size was increased from 2,000 study subjects, initially foreseen, to a total of over 3,000 study subjects.

In recent years, it has become clear that genetic associations of polymorphisms with phenotypes (e.g. circulating hormone levels or breast cancer risk), if they exist, generally tend to be rather weak. Furthermore, the trend is towards more exhaustive genotyping and studies of associations at the level of multiple SNPs, combined into haplotypes. In our original proposal we had anticipated stronger associations, but for fewer SNPs. Fortunately, with progressively decreasing costs of genotyping assays we were able to perform exhaustive haplotype analyses, based on a much larger number of SNPs than originally foreseen.

Our study relating breast cancer risk to prediagnostic serum IGF-I levels of IGFBP-3 is by the largest study of this type conducted so far, and its report in the literature Sabina Rinaldi et al.(1) will likely have a major impact on current thinking about the role of circulating IGF-I (or possible lack of it?) in breast cancer development.

Our study relating circulating IGF-I, IGFBP-3 and breast cancer risk to genetic polymorphisms in genes involved in IGF-I synthesis and physiology is currently also the largest, and amongst the most comprehensive, conducted so far. These studies have shown a number of new genetic associations of great potential interest (to be confirmed by independent studies, however). In addition, our study confirms, on a large scale, some earlier observations made in other studies, notably relating polymorphisms in the *IGF1* and *IGFBP3* genes to breast cancer risk and circulating IGFBP-3, respectively.

Five articles have been prepared to report major study findings in the scientific literature, and a further two papers are planned to be prepared within the coming six months.

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IGF-I, IGFBP-3 AND BREAST CANCER RISK IN WOMEN: THE EUROPEAN PROSPECTIVE INVESTIGATION INTO CANCER AND NUTRITION (EPIC)

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Running title: IGF-I and breast cancer risk in women

Key words: breast cancer, IGF-I, IGFBP-3, cohort studies

Abbreviations used: European Prospective Investigation into Cancer and nutrition (EPIC); Body mass index (BMI); Enzyme-linked immunosorbent assays (ELISA); Odds ratio (OR); Confidence interval (CI)

Journal Category: Epidemiology

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Abstract

Blood concentrations of insulin-like growth factor-I (IGF-I) and its major binding protein, insulin-like growth factor binding protein-3 (IGFBP-3) have been recently associated to breast cancer risk, above all in women who developed breast cancer at relatively young age. Studies published so far, however, were relatively small and odds ratio (OR) estimates rather imprecise. We present the results of a very large prospective case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) on IGF-I, IGFBP-3 and risk of breast cancer, which includes 1,195 incident breast cancer and 2,321 matched control subjects. Overall, IGF-I and IGFBP-3 were associated to a significant increase in breast cancer risk in older women who developed breast cancer after 50 years of age (highest *vs* lowest quintile OR 1.30 [0.98-1.72], p = 0.02, and 1.38 [1.02-1.86, p = 0.03, respectively), but no relationship was observed in young pre-menopausal women (highest *vs* lowest quintile OR was 1.08 [0.65-1.79], p = 0.86 for IGF-I, and 1.03 [0.58-1.84], p = 0.93 for IGFBP-3). A mild association of IGF-I and IGFBP-3 and androgen and estrogen concentrations with breast cancer risk was observed in post-menopausal women, but not in pre-menopausal women. This study does not confirm previous findings of an association of IGF-I and IGFBP-3 concentrations with breast cancer risk in young women, but gives support for the involvement of those hormones in breast cancer aetiology in older women.

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Introduction

Insulin-like growth factor-I (IGF-I) is a polypeptide hormone consisting of 70 amino acids that is involved in multiple cellular responses related to growth, including synthesis of DNA, RNA and cellular proteins (1,2). IGF-I has been characterized as a systemic hormone with mitogenic and anti-apoptotic properties, actions that may influence the proliferative effect of many cell types including breast epithelial cells (3). Laboratory studies have also shown that IGF-I may act synergistically with estrogens to stimulate proliferation of breast cancer cells (4).

IGF-I is primarily synthesized in the liver in response to growth hormone production, but its availability in tissues also depends on circulating concentrations and tissue production of its binding proteins (IGFBP), the most abundant type being IGFBP-3 to which about 90% of IGF-I is bound. Apart from its role of regulation of IGF-I bioavailability, IGFBP-3 acts itself in a paracrine and autocrine manner, and has anti-mitogenic and pro-apoptotic effects on several cell lines, including breast epithelial cells (5).

Elevated IGF-I levels have been associated with an increased risk of several malignancies such as prostate, colon and breast cancers (6-8). Recently, a number of prospective cohort studies (8-11) and case-control studies (12,13) have shown an increased risk of breast cancer in women with elevated circulating levels of IGF-I, especially in women who developed cancer at a relative young age (8,9). A recent meta-analysis on all published prospective and case-controls studies (14) found an overall doubling of relative risk of breast cancer for young women (aged less than 50), mainly pre-menopause, having the highest category of IGF-I concentrations, but no association among older women, mainly in post-menopausal status. This observation was supported by another meta-analysis that showed that premenopausal cases have on average 56.7% higher IGF-I levels than controls (15). The heterogeneity of breast cancer risk according to circulating levels of IGF-BP3, however, has been more pronounced with some studies showing a direct relationship of risk with IGFBP-3 concentrations (9-11,13,16), while other studies showed either a protective effect (8,12), or no association (17).

In this paper, we present results from a large case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) – a multi-centre prospective study aimed at investigating the relationships between nutrition and other lifestyle factors, metabolism, genetic predisposition and cancer risk. In total, our study includes 1,195 incident breast cancer and 2,321 matched control subjects, and thus is several times larger in size than all previously published cohort studies combined.

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Material and methods

Study population

Between 1992 and 1998, extensive standardized questionnaire data on diet and non-dietary variables, anthropometric measurements, and blood samples were collected from about 250,000 women and 150,000 men living around 23 research centers spread over 10 western European countries (18). Questionnaires included detailed questions about habitual diet and physical activity, lifetime history of tobacco smoking and consumption of alcoholic beverages, history of previous illness and surgical operations, and for women, menstrual and reproductive history, current and past use of oral contraceptives (OC), postmenopausal hormone replacement therapy (HRT),

Height, weight, and waist and hip circumferences were measured according to standardized protocols, in light dressing, while women visited one of the recruitment center. In part of the Oxford cohort, height, weight and body circumferences were self-reported. All measurements were reported to the nearest centimeter (height, body circumferences), and to the nearest kilogram (weight).The present study includes breast cancer case and control subjects from 19 recruitment centers in 8 of the participating countries: France, the Netherlands, the United Kingdom, Germany, Spain, Italy, Denmark and Greece. Norway was not included in the present study because blood samples have only recently been collected on a sub-sample of cohort participants, and so far only very few cases of breast cancer have cumulated after blood collection; Sweden was not included because, for post-menopausal women, independent studies on breast cancer risk and endogenous hormones have been, or are being, conducted separately, and, for pre-menopausal women, limited questionnaire information was available on past and current OC use, and phase of menstrual cycle.

Collection and storage of blood samples

In France, the Netherlands, the United Kingdom, Germany, Spain, Italy and Greece blood samples were collected according to a standardized protocol (18). From each subject, 30 ml of blood was drawn using 10-ml Safety Monovettes (Sartstedt, Nümbrecht, Germany). Filled syringes were kept at 5-10°C, protected from light, and transferred to a local laboratory for further processing and aliquoting. For the preparation of plasma, buffy coat and red cells, trisodium citrate was used as anticoagulant in two out of three syringes, and one dry syringe was used to prepare serum. After centrifugation (1550g for 20 minutes), blood fractions (serum, plasma, red cells, buffy coat) were aliquoted in 28 plastic straws of 0.5 ml each (12 plasma, 8 serum, 4 erythrocytes, 4 buffy coat for DNA), which were heat-sealed and stored under liquid nitrogen (-196°C). One half of the 28 aliquots were stored locally, and the other half centrally at the International Agency for Research on Cancer (IARC). In Denmark, non-fasting blood samples were drawn, and serum, plasma, red cells

or buffy coat were aliquotted into 1-ml tubes, stored in the vapor phase in liquid nitrogen containers (-150°C).

Follow-up for cancer incidence and vital status

In Denmark, the Netherlands, the United Kingdom, Spain and Italy, incident cancer cases were identified through record linkage with regional cancer registries. In Germany, France and Greece follow-up was based on a combination of methods, including health insurance records, cancer and pathology registries, and active follow-up through study subjects and their next-of-kin. Data on vital status in most EPIC study centers were collected from mortality registries at the regional or national level, in combination with data collected by active follow-up (Greece). For each EPIC study center, closure dates of the study period were defined as the latest dates of complete follow-up for both cancer incidence and vital status (dates varied between centers).

Determination of menopausal status at blood donation

Women were considered as pre-menopausal when they reported having had regular menses over the past twelve months, or when they were less than 42 years of age. Women were considered as post-menopausal when they reported not having had any menses over the past 12 months, or when they reported bilateral ovariectomy. Women with missing or incomplete questionnaire data, or with reported previous hysterectomy, were considered postmenopausal only if they were older than 55 years. When women had missing or incomplete questionnaire data, or with reported previous hysterectomy, and were between 42 and 55 years of age, they were classified as peri-menopausal/unknown.

Selection of case and control subjects

Case subjects were selected among women who developed breast cancer after their recruitment into the EPIC study, and before the end of the study period (defined for each study center by the latest end-date of follow-up). Women who used any hormone replacement therapy at the time of blood donation, or any exogenous hormones for contraception or medical purposes, and who had previous diagnosis of cancer (except non-melanoma skin cancer) were excluded from the study.

A total of 416 incident cases of breast cancer were identified among women classified as pre-menopausal at the time of blood donation; of these, 46 had a carcinoma *in situ* and all others (N = 370) had an invasive tumor. A total of 677 incident cases were identified as post-menopausal, 63 of which had a carcinoma *in situ* and all others had an invasive tumor. A total of 102 cases were identified as peri-menopausal/unknown, 5 had carcinoma *in situ*.

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For each case subject with breast cancer, two control subjects were chosen at random among appropriate risk sets consisting of all cohort members alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. An incidence density sampling protocol for control selection was used, such that controls could include subjects who became a case later in time, while each control subject could also be sampled more than once. Matching characteristics were the study center where the subjects were enrolled in the cohort, menopausal status (pre-menopausal, post-menopausal, peri-menopausal/unknown), age (± 6 months) at enrolment, follow-up time since blood collection, time of the day at blood collection, fasting status (< 3 hours; 3-6 hours, > 6 hours) (19), and, for pre-menopausal women, phase of menstrual cycle ('early follicular' (days 0-7 of the cycle), 'late follicular' (days 8-11), 'peri-ovulatory' (days 11-16), 'midluteal' (days 20-24) and 'other luteal' (days 17-19 or 25-40) (20).

All participants had given their written consent for future analyses of their blood samples and the Internal Review Board (IRB) at IARC approved the hormone analyses.

Laboratory assays:

All hormone assays were performed at the laboratory of the Nutrition and Hormones Group, at IARC. IGF-I and IGFBP-3 were measured by enzyme-linked immunosorbent assays (ELISA) from Diagnostic System Laboratories (DSL, Webster, Texas). IGF-I assays included an acid-ethanol precipitation of IGF-I binding proteins, to avoid interference of IGFBPs with the IGF-I assay. Measurements were performed on never thawed serum sample aliquots. The laboratory personnel performing the assays were blinded as to the case-control status of the study subjects. Cases and matched controls were always analyzed in the same analytical batch. The mean intra-batch and inter-batch coefficients of variation were 6.3% and 14.7% respectively for IGF-I, and 8.2% and 13.0% respectively, for IGFBP-3.

IGF-I and IGFBP-3 were measured on 801 breast cancer cases and 1,554 matched controls between March-August 2002 (first wave), and on 394 breast cancer cases and 767 matched controls between January and March 2004 as a result of a subsequent follow-up.

On the same samples, measurements of testosterone, androstenedione, dehydroepiandrosterone sulphate (DHEAS), estrone, estradiol and progesterone (in pre-menopausal women only) and sex-hormone binding globulin (SHBG) were also performed, as part of nested case-control studies on hormones and breast cancer conducted within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (19,20). Those measurements were performed by using direct radioimmunoassay, previously validated against a reference method, while free testosterone and free estradiol concentrations were calculated from the absolute concentrations of each of the

steroids and SHBG using mass action equations, and assuming a constant serum albumin concentration of 43 g/l published previously (21).

Statistical analyses:

In all analyses, measurements of IGF-1 and IGFBP-3 were transformed using the natural logarithm to normalize their distributions. An analysis of variance, adjusting for age, was used to examine study centre and batch as a determinant of measured hormone levels. Correlations between IGF-1 and IGFBP-3 adjusting for age, case-control status and batch were calculated with Pearson partial correlation coefficient for all women.

A pairwise t-test was used to test for mean case-control differences in age at blood donation, age at diagnosis, age at first full term pregnancy, number full term pregnancies, age at menarche, BMI (calculated as kilograms divided by the square of the height expressed in meter), height, waist, weight, parity, percentage of past hormone users, and hormone levels. Odds ratios (OR) for disease by quintile level of the hormone variables were estimated by conditional logistic regression models using the SAS 'PHREG' procedure. The quintile cut-off points were based on the hormone variable distributions of the controls. For IGFBP-3, specific cut-off points were chosen for each part of the study because of a sudden change in absolute levels in measurements obtained in the second part of the study (follow-up). Likelihood ratio tests were used to assess linear trends in ORs over the quintiles, scoring the categories according to the quantitative score 1, 2, 3, 4 and 5 for quintile categories. All statistical tests and corresponding *P*-values were two-sided, and *P*-values <0.05 were considered statistically significant. Heterogeneity of ORs between the different EPIC centers/countries was assessed by chi-square tests.

Multivariate logistic regression was used to estimate ORs adjusted for possible confounders other than those controlled for by the matching criteria, including age at first full term pregnancy, number of full term pregnancies, age at menarche, BMI, parity, and past use of HRT or OC and age at menopause (for post-menopausal women). Adjustment of IGF-I for IGFBP-3 was made by a two-step procedure. First, in a linear regression model, values for IGF-I were regressed on IGFBP-3 level separately for each part of the study and the residuals of these regressions were categorized into quintiles. Then, ORs were estimated for the quintiles of the residuals by multivariate conditional logistic regression models. Interactions between IGF-I, IGFBP-3 and sex steroids were examined in the regression analysis by fitting a logistic regression model with two independent variables of interest plus a product term of the two variables.

All statistical analyses were done using the Statistical Analysis System (SAS) software package, version 8 (SAS Institute, Cary, NC, USA).

APPENDIX 1

Results

Basic characteristics of case and control subjects at recruitment are presented in Table 1. Compared to the control subjects, cases were taller (161.0 [150.6-172.0] *vs* 160.5 [150.0-172.0], p = 0.05), had fewer children (2.3 [1.0-4.0] *vs* 2.5 [1.0-5.0], p = 0.002), and were younger at the time of their first full term pregnancy [25.0 (20.0-34.0) *vs* 25.3 [19.0-33.0], paired t-test p = 0.01). In post-menopausal women, cases had a higher BMI compared to the controls (27.2 [21.1-36.0] *vs* 26.8 [20.6-35.6], p = 0.05). IGF-I and IGFBP-3 concentrations were about 2% higher in cases than in controls (p = 0.06 and p = 0.02 for IGF-I and for IGFBP-3, respectively).

As expected, IGF-I concentrations were markedly positively correlated with IGFBP-3 concentrations (Pearson r = 0.48, p<0.0001), very mildly positively correlated to androgen concentrations (Pearson r between 0.10 and 0.15, p<0.0001) and inversely correlated to SHBG (r = -0.20, p<0.0001). No correlation was observed between IGF-I and estrogen concentrations. IGF-I was inversely correlated with age both in post-menopausal women (Pearson r = -0.13, p<0.0001) and in pre-menopausal women (r = -0.27, p<0.0001). IGFBP-3 was found to be inversely correlated to SHBG (r = -0.24, p<0.0001), while no correlation was observed with both androgens and estrogens. IGFBP-3 was only very slightly negatively correlated to age in pre-menopausal women (r = -0.09, p = 0.002), while no statically significant relationship was observed for pre-menopausal women and peri/unknown. In the control population, a non-linear relationship between BMI and serum levels of IGF-I was found, while a positive correlation was observed between BMI and IGFBP-3 in both pre and post-menopausal women (22).

No heterogeneity in ORs estimates for both IGF-I and IGFBP-3 (p = 0.79 and p = 0.45, respectively) was observed among the 8 study countries on the whole population, as well as on the analyses restricted to case subjects who were younger than 50 (p = 0.60 for IGF-I and p = 0.94 for IGFBP-3), or older than 50 at diagnosis (p = 0.69 for IGF-I, and p = 0.50 for IGFBP-3).

Logistic regression analysis on the whole population suggested an overall increase of breast cancer risk with increasing IGF-I and IGFBP-3 concentrations, even though not statistically significant. The ORs for the highest *vs* lowest quintile of IGF-I and IGFBP-3 were 1.26 [95% CI 0.98-1.61], p_{trend}= 0.22 and 1.25 [0.96-1.62], p_{trend}= 0.08, respectively, for a model adjusted for BMI, age at first full term pregnancy, number of full term pregnancies and age at menarche. Residuals of IGF-I on IGFBP-3, as an indication of IGF-I, which is not bound to IGFBP-3, did not show any association with breast cancer risk.

When the analysis was restricted to cases that were younger than 50 at time of cancer diagnosis, no association was found for IGF-I and IGFBP-3 concentrations with breast cancer risk (Table 2). Highest *vs* lowest quintile OR was 1.08 [0.66-1.75], p = 0.82 for IGF-I, and 1.06 [0.61-1.83], p = 0.97 for IGFBP-3. Adjustment for BMI, as further adjustments for age at first full term pregnancy,

number of full term pregnancies, age at menarche and previous use of oral contraceptives did not have effect on the association. This lack of association was also observed when restricting analyses to women who were both pre-menopausal and less than 50 at diagnosis of breast cancer, or to women who were both pre-menopausal and aged less than 50 at time of blood donation.

In women who had a diagnosis of breast cancer after 50 years of age, a direct, statistically significant relationship of breast cancer risk with increasing IGF-I and IGFBP-3 concentrations was observed (Table 3). For IGF-I, the OR for highest *vs* lowest quintile was 1.39 [1.05-1.82], p = 0.01. Adjustment for BMI and for age at first full term pregnancy, number of full term pregnancies, age at menarche and previous use of oral contraceptives, previous use of HRT, age at menopause and time since menopause showed no effect. For IGFBP-3, the OR for highest *vs* lowest quintile was 1.47 [1.09-1.96], p = 0.005. Adjustments for BMI, age at full term pregnancy, number of full term pregnancies, age at menarche, previous use of oral contraceptives, previous use of HRT, age at menopause and time since menopause showed very minor effect on the association. Similar results were obtained using the same adjusted model when restricting the analyses to women who were post-menopausal at blood donation (highest *vs* lowest quintile OR 1.31 [0.95-1.83], p = 0.04, and OR 1.48 [1.06-2.1], p = 0.03, for IGF-I and IGFBP-3, respectively), who were above 55 at diagnosis (highest *vs* lowest quintile OR 1.33 [0.96-1.85], p = 0.03, and 1.62 [1.16-2.26], p = 0.01, for IGF-I and IGFBP-3, respectively) or above 60 at diagnosis (highest *vs* lowest quintile OR 1.44 [0.95-2.18], p = 0.03, and 1.92 [1.26-2.94], p = 0.003, for IGF-I and IGFBP-3, respectively). Residuals of IGF-I for IGFBP-3 did not show any association with breast cancer risk.

Table 4 shows the associations of breast cancer risk with combined distributions of steroid hormones, SHBG and IGF-I and IGFBP-3 in post-menopausal women. In this population, ORs for IGF-I in combination with androstenedione, and estradiol were slightly higher than the ORs for each individual hormone. The same effect could be observed for IGFBP-3 and testosterone, androstenedione, DHEAS and estradiol. None of these interactions however, resulted to be statistically significant. In pre-menopausal women, no combined effect of IGF-I and IGFBP-3 with any steroid hormones or SHBG was observed (data not shown).

Discussion

In this prospective study, which is the largest ever published on the relationship between breast cancer risk, IGF-I and IGFBP-3 concentrations in blood, we observed an overall significant increase in breast cancer risk in women with relatively high IGF-I and IGFBP-3 serum concentrations. However, when stratifying by age, a statistically significant increase was mainly found in women who developed breast cancer at a relatively older age (above 50), while no significant association

was found in women who developed breast cancer at a relatively young age (before 50) for both peptides. Residuals of IGF-I on IGFBP-3 showed no relationship at all with breast cancer risk.

The results of the current study contrast somewhat with recent findings in both prospective and case-control studies (9,10). Recently, three meta-analysis on breast cancer risk in women and IGF-I concentrations in blood have been published (14,15,23). In these meta-analyses, an overall increase in risk with increasing IGF-I concentrations in pre-menopausal women has been observed [$OR_{highest\ vs\ lowest}$ = 1.74, 95% CI = 0.97, 3.13; in the analysis by Shi et al (15) $OR_{high\ vs\ low}$ = 1.39, 95%5 CI = 1.13, 1.69 in the analysis by Sugumar et al (23), and $OR_{highest\ vs\ lowest}$ = 1.96, 95% CI = 1.28, 2.99 in the analysis by Renehan et al (14) , while no risk has been observed in postmenopausal women. A much broader discrepancy in results has been observed for IGFBP-3 concentrations and breast cancer risk in pre-menopausal women (15,23), while no association was globally found for post-menopausal women (15).

In the present study, no association between IGF-I and IGFBP-3 concentrations in blood and breast cancer risk in young women was observed. This lack of association was present also when the analyses were restricted to women who were less than 50 at diagnosis, who were pre-menopausal and aged less than 50, or to women who were less than 50 at recruitment in the study. Those data do not confirm the overall evidence of an implication of IGF-I and IGFBP-3 in an early manifestation of the disease (14,15,23).

An indication for an increase in risk with increasing IGF-I concentrations in older women have been observed in only one prospective study, (17) and few case-control studies (13,24). It has been shown that menopausal women with breast cancer have frequently more estrogens-IGF receptor positive tumors compared to pre-menopausal women (24). However, in those studies, the number of breast cancer cases in post-menopausal women was relatively small.

The current study has the advantage of having a very large number of breast cancer cases for both young and relatively older women. Its prospective design, furthermore, rules out the fact that circulating hormone levels might be influenced by the presence of the disease {refs}. Another substantial advantage of the present study is that, as part of a whole study, measurements of IGF-I and IGFBP-3 have been done using the same methods, therefore avoiding the problem of comparison results obtained by different assays, as for meta-analyses. A relative disadvantage of the study might be its heterogeneity in population, and its multicentric component. However, standardized protocols were followed for recruitment, blood collection and questionnaire data. Moreover, no heterogeneity of ORs estimates has been observed among countries. Another disadvantage of the present study might be that blood has been collected only once over-time. However, it has been observed that reproducibility within-subjects of serum IGF-I and IGFBP-3 concentrations over a one-year period is quite high (intra-class correlations above 0.73 for cases and

controls for IGF-I, and above 0.84 for IGFBP-3, refs Lukanova), therefore a single blood collection is rather representative of the exposure over a relatively long period of time.

It has been recently shown that the association between concentrations of IGF-I, and above all IGFBP-3, and the increase in breast cancer risk might be different depending on the assays used {refs}. In the current study, IGF-I and IGFBP-3 have been measured by using an ELISA assay by DSL. These assays have also been used in some of the published studies, in which a relationship has been found between IGF-I concentrations and the increase in risk of several cancers, (6,25), including breast cancer (8). In our laboratory, the DSL ELISA assay has been used routinely giving good results as to the lot-to-lot reproducibility of measurements (correlation coefficients between IGF-I concentrations obtained by ELISA assays over-time -which periods??, different lot numbers, how many samples- were found to be above ??). In addition, measurements on serum samples obtained by this assay were found to be highly correlated with those obtained by other IGF-I assays, as immunoradiometric assays, produced either by the same company, or by different companies (data not published), and with-in-house methods (26). Those high correlation coefficients rule out the hypothesis that differences between assays, or difference between different lot numbers of the same assays might have affected the results of the study. Absolute levels were also comparable among methods and assay lots.

IGFBP-3 measurements were found to be somewhat less robust than the measurements of IGF-I. IGFBP-3 is a protein that is present in blood in a mixture of glycosilated and non-glycosilated forms, and is subject to the activity of different proteases in blood, and therefore it can be present in blood more or less intact forms with different affinities for IGF-I {refs}. It has been speculated that different assays for IGFBP-3 measurements may measure different forms of this protein, and therefore the association to breast cancer risk may vary depending on the assay used (27). This heterogeneity of results has also been observed in the meta-analyses published by Shi et al (15). However, a constant direct association of IGFBP-3 with breast cancer risk was generally observed in older women when this protein was measured by ELISA (15), and this is confirmed by the results of the present study.

In our laboratory, IGFBP-3 measurements by DSL ELISA were found to be high reproducible over-time between different lot numbers (r>???), but absolute levels tended to change quite substantially between different lots of production. A sudden change in absolute levels was observed over a one-year period, when the analyses of the second part of the study were performed, so cut-off points for IGFBP-3 concentrations had to be chosen differently in the two parts of the study. However, since cases and matched controls were measured within the same assay kit, this change in absolute levels did not influence the case-control comparison with breast cancer risk, and the very

high correlation between absolute levels from different lots of assays did not influence the ranking of the subjects according to their levels of IGFBP-3.

No joint effects of IGF-I and IGFBP-3 with steroids and SHBG concentrations in blood have been observed in the present study in young women, but a suggestion of interaction between IGF-I and IGFBP-3 with steroids has been observed in post-menopausal women, even though this interaction was not statistically significant. This partially confirms the results of a recent case-control study, where a synergistic effect was found for IGF-I and IGFBP-3, and estrone and estradiol in both pre- and post-menopausal women (7). In that study, the interactions between IGF-I, IGFBP-3 and steroids resulted to be highly significant, however, considering the case-control design of the study, the influence of the disease and of treatments on hormone concentrations cannot be ruled out. To our knowledge, ours is the first prospective nested case-control study ever published exploring the synergistic effects of sex steroids (and SHBG) with IGF-I and IGFBP-3, so our results need to be confirmed in further studies.

In conclusion, the present study does not confirm previous observations of an increase in breast cancer risk with increasing IGF-I and IGFBP-3 concentrations in young women, but it does strengthen the association between those two proteins and breast cancer risk in older women.

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Table 1. Basic characteristics of the study participants, all study centres combined; The European Prospective Investigation into Cancer and Nutrition (EPIC)

Characteristics	Cases	Controls	P value
Number of subjects	1195	2321	
Menopausal status at blood donation			
Pre	416	815	
Post	677	1311	
Peri	102	195	
Years between blood donation and diagnosis	2.86 [0.09-6.27]	-	-
Age at diagnosis[1]	57.0 [43.0-72.0]	-	-
Age at first pregnancy[1]	25.0 [20.0-34.0]	25.3 [19.0-33.0]	0.01[3]
Number of full term pregnancies	2.3 [1.0-4.0]	2.5 [1.0-5.0]	0.002[3]
Age at menarche [1]	13.0 [11.0-16.0]	13.1 [11.0-16.0]	0.20[3]
Body Mass Index [1]	26.3 [20.3-34.7]	26.2 [20.2-34.9]	0.50[3]
Pre	25.0 [19.7-32.9]	25.3 [19.9-33.9]	0.19[3]
Post	27.2 [21.1-36.0]	26.8 [20.6-35.6]	0.05[3]
Peri/unknown	25.9 [19.6-32.5]	26.0 [20.1-35.3]	0.72[3]
Body Height [1]	161.0 [150.6-172.0]	160.5 [150.0-172.0]	0.05[3]
Body Weight [1]	68.2 [51.7-89.5]	67.5 [51.9-89.0]	0.08[3]
Waist [1]	82.9 [66.0-103.0]	82.4 [67.0-104.0]	0.21[3]
Waist-hip ratio [1]	0.80 [0.70-0.91]	0.80 [0.70-0.91]	0.89[3]
Parous (%)	85	86	0.82[4]
Previous us of HRT (%)	13	14	0.70[4]
Previous OC use (%)	44	46	0.45[4]
IGF-I (ng/ml) [2]	230.6 [226.2-235.1]	226.2 [223.1-229.3]	0.06[3]
IGFBP-3 (ng/ml) [2]	3422 [3349-3495]	3353 [3302-3405]	0.02[3]

[1] means (5^{th} – 95^{th} percentile range)

[2] geometric mean (95%CI)

[3] Paired t-test

[4] Mantel-Haenszel Chi-square

APPENDIX 1

Table 2. Odds ratios of breast cancer [95% confidence intervals] by quintiles of IGF-I and IGFBP-3 in cancer cases with age at diagnosis less or equal to 50, and their matched controls

		Quintiles					P trend•
		1	2	3	4	5	
IGF-I	Crude[1]	**1.00**	**1.26(0.81-1.94)**	**1.05(0.67-1.64)**	**0.98(0.61-1.56)**	**1.08(0.66-1.75)**	**0.82**
	Adjusted[2]	1.00	1.25 (0.81-	1.07 (0.68-1.69)	0.99 (0.62-1.59)	1.09 (0.67-1.77)	0.89
	Adjusted[3]	1.00	1.26 (0.80-	1.03 (0.64-1.65)	0.98 (0.60-1.60)	1.08 (0.65-1.79)	0.85
	No. of cases and	**57/117**	**69/115**	**58/117**	**54/117**	**60/116**	
	Residuals[2]	1.00	1.04 (0.64-	0.90 (0.55-1.45)	0.93 (0.56-1.53)	0.83 (0.48-1.43)	0.42
	No. of cases and controls	62/107	60/106	54/107	55/106	51/106	
IGFBP-3	Crude[1]	**1.00**	**1.02 (0.64-1.60)**	**1.04 (0.63-1.73)**	**0.90 (0.52-1.53)**	**1.06 (0.61-1.83)**	**0.97**
	Adjusted[2]	1.00	1.02 (0.65-	1.06 (0.64-1.76)	0.92 (0.53-1.58)	1.09 (0.62-1.90)	0.90
	Adjusted[3]	1.00	1.03 (0.64-	1.07 (0.63-1.82)	0.88 (0.50-1.55)	1.03 (0.58-1.84)	0.93
	No. of cases and controls	57/109	58/109	57/104	54/114	58/102	

• Linear trends in ORs over the quintiles by scoring the categories according to the quantitative score 1,2,3,4 and 5 for quintile categories

[1] analysis matched on EPIC recruitment centre, age at blood donation, follow-up time, menopausal status, phase of menstrual cycle, time of the day at blood donation, and fasting status

[2]Further adjustment for BMI

[3] Further adjustment for age at first full term pregnancy, number of full term pregnancies, age at menarche and previous use of oral contraceptives

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Table 3. Odds ratios of breast cancer [95% confidence intervals] by quintiles of IGF-I and IGFBP-3 in cancer cases with age at diagnosis above 50, and their matched controls

		Quintiles					P trend•
		1	2	3	4	5	
IGF-I	**Crude[1]**	**1.00**	**0.89 (0.68-1.17)**	**0.98 (0.75-1.28)**	**1.11 (0.85-1.45)**	**1.39 (1.05-1.82)**	**0.01**
	Adjusted[2]	1.00	0.88 (0.67-1.15)	0.97 (0.75-1.27)	1.10 (0.84-1.45)	1.36 (1.04-1.80)	0.01
	Adjusted[3]	1.00	0.84 (0.64-1.11)	0.96 (0.73-1.26)	1.07 (0.81-1.41)	1.30 (0.98-1.72)	0.02
	Number of cases and controls	172/346	151/345	166/346	184/346	221/345	
	Residuals[2]	1.00	0.93 (0.71-1.23)	0.80 (0.60-1.07)	0.93 (0.69-1.25)	1.12 (0.83-1.50)	0.49
	Number of cases and controls	179/324	170/324	146/324	170/324	197/325	
IGFBP-3	**Crude[1]**	**1.00**	**0.86 (0.65-1.12)**	**0.97 (0.74-1.28)**	**1.07 (0.81-1.41)**	**1.47 (1.09-1.96)**	**0.01**
	Adjusted[2]	1.00	0.86 (0.66-1.13)	0.96 (0.73-1.26)	1.04 (0.79-1.38)	1.43 (1.06-1.92)	0.01
	Adjusted[3]	1.00	0.86 (0.66-1.14)	0.95 (0.71-1.25)	1.02 (0.76-1.36)	1.38 (1.02-1.86)	0.03
	Number of cases and controls	172/331	151/344	163/333	172/325	205/292	

• Linear trends in ORs over the quintiles by scoring the categories according to the quantitative score 1,2,3,4 and 5 for quintile categories

[1] analysis matched on EPIC recruitment centre, age at blood donation, follow-up time, menopausal status, time of the day at blood donation, and fasting status

[2] further adjustment for BMI

[3] further adjustment for age at first full term pregnancy, number of full term pregnancies, age at menarche and previous use of oral contraceptives, previous use of HRT, age at menopause, time since menopause

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Table 4: ORs and 95% confidence intervals for IGF-I, IGFBP-3, steroids hormones and their interactions among postmenopausal women[1]

Variable	IGF-I		Int. Test[2]	IGFBP-3		Int Test[2]
	low	high		low	high	
Testosterone						
low[3]	1.00	1.21 [0.91-1.60]	0.40	1.00	1.46 [1.09-1.96]	0.67
high	1.61 [1.21-2.15]	1.68 [1.27-2.21]		1.58 [1.18-2.12]	1.97 [1.47-2.62]	
Androstenedione						
low	1.00	1.11 [0.84-1.48]	0.76	1.00	1.49 [1.11-2.01]	0.47
high	1.29 [0.97-1.71]	1.50 [1.14-1.97]		1.44 [1.07-1.93]	1.88 [1.40-2.51]	
DHEAS						
low	1.00	1.24 [0.93-1.65]	0.49	1.00	1.58 [1.15-2.10]	0.88
high	1.64 [1.24-2.18]	1.65 [1.26-2.16]		1.69 [1.26-2.28]	2.02 [1.51-2.71]	
SHBG						
low	1.00	0.92 [0.70-1.21]	0.16	1.00	1.18 [0.88-1.59]	0.59
high	0.69 [0.52-0.90]	0.98 [0.73-1.32]		0.76 [0.56-1.01]	1.18 [0.86-1.63]	
Estrone						
low	1.00	1.31 [0.98-1.74]	0.43	1.00	1.55 [1.15-2.09]	0.63
high	1.47 [1.09-1.98]	1.56 [1.16-2.09]		1.43 [1.05-1.96]	1.73 [1.29-2.31]	
Estradiol						
low	1.00	1.11 [0.84-1.48]	0.61	1.00	1.49 [1.10-2.01]	0.84
high	1.70 [1.26-2.29]	1.90 [1.43-2.53]		1.84 [1.35-2.51]	2.13 [1.59-2.87]	

[1] Conditional regression analysis matched on EPIC recruitment centre, age at blood donation, follow-up time, menopausal status, time of the day at blood donation, and fasting status.

[2] **Interaction test**

[3] low, lower than the median; high, higher than or equal to the median

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Anthropometric Indices of Adiposity and Serum IGF-I Levels; Further support of a Non-Linear Relationship with BMI in the EPIC cohort.

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Running title: Obesity and serum levels of IGF-I and IGFBP-3

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Abstract

Elevated serum levels of insulin-like growth factor- I (IGF-I), measured as absolute concentrations or expressed relative to levels of its major plasmatic binding protein, IGFBP-3, have been associated with increased risks of several types of cancer. However, it is still relatively unclear, to what extent circulating IGF-I and IGFBP-3 levels are influence by lifestyle factors.

In a large cross-sectional study of 2,139 healthy female participants in the European Prospective Investigation into Nutrition and Cancer (EPIC), we examined the relationships between anthropometric indices, and selected characteristics (age, menopausal status, age at menarche, parity, former oral contraceptive (OC) and hormone replacement therapy (HRT) use, smoking, and alcohol consumption) and serum levels of IGF-I and IGFBP-3. The multivariate analyses performed with the general linear model revealed that after adjustments for age and laboratory batch; body weight, body mass index (BMI), and waist and hip circumference showed a non-linear relationship with serum levels of IGF-I ($p_{association} < 0.05$). The non-linearity was most evident for BMI ($p_{association} < 0.0001$), with the highest serum levels of IGF-1 in the third quintile of BMI, i.e 24.6-26.6 kg/m^2. Body height was inverse, while body weight, BMI, waist circumference, and waist, hip, ratio (WHR) was positively associated with IGFBP-3 ($all\ p_{trend} < 0.05$). Similar associations were displayed after stratification by menopausal status. Alcohol consumption was inversely related to IGF-1 and positively to IGFBP-3. None of the other life style factors showed a statistical significant association with the hormone levels. In summary, our study supports the notion of a non-linear relationship between BMI and serum IGF-I levels among healthy women.

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Introduction

Elevated serum levels of insulin-like growth factor- I (IGF-I), measured as absolute concentrations or expressed relative to levels of its major plasmatic binding protein, IGFBP-3, have been associated with increased risk of several common cancers such as breast, colorectal, prostate, and lung. On the other hand, risk of cardiac disease seems to vary inversely with IGF-1 levels (1).

Circulating levels of IGF-1 and IGFBP-3 vary considerably between normal individuals. In an earlier review, we have described how restriction of nourishment in animal studies, and chronically undernourished states such as anorexia nervosa, are associated with strongly reduced circulating IGF-I levels. IGF-I levels are also moderately reduced in obese, compared with normal weight, subjects (2). These observations suggest a non-linear relationship of serum IGF-I with body weight or indices of adiposity. Except for nutrition, it is unclear what other lifestyle factors may influence the IGF-1 levels (3).

We conducted a cross-sectional analysis among more than 2,000 healthy women to examine the association between anthropometric indices of adiposity and selected lifestyle factors with serum levels of IGF-I and IGFBP-3. We especially wanted to examine if the hypothesized non-linear relationship between BMI and IGF-I levels, could be supported in this data.

Materials and Methods

Study population. The study is comprised of women from the European Prospective Investigation into Cancer (EPIC) – a large, multicentre cohort of about 370,000 women and 150,000 men, recruited between 1992 and 1998 from 10 western European countries (4). The women in the present study were selected from 2,278 control subjects of a nested case-control study on the relationship between endogenous hormones and breast cancer (5, 6). The

women included were from 19 recruitment centers in eight countries, i.e. Denmark, the Netherlands, the United Kingdom (UK), France, Germany, Spain, Italy and Greece.

The women had their body height, waist and hip circumferences measured to the nearest 0.5 centimeter and weight to the nearest 0.1 kilogram, (allowing us to calculate body-mass-index (BMI) as weight in kilograms divided by the square of height in meters and waist-hip ratio (WHR) as waist circumference by hip circumference). The protocols for how the measurements were obtained varied slightly for some centers. Details are described elsewhere (7). The women completed standardized questionnaires concerning among other things health factors, menstrual and reproductive history, oral contraceptive (OC) and hormone replacement therapy (HRT) use, lifetime history of tobacco smoking and consumption of alcoholic beverages. Only women who did not use OC or HRT at the blood draw were eligible. We excluded 15 women with a diagnosis of cancer (except non-melanoma skin cancer), 14 women taking insulin as a medication for diabetic conditions, 106 women recruited in Oxford, UK, who had self-reported and 4 women with missing values for anthropometric data, leaving 2,139 women for the present overall analysis.

Women who reported to have had a bilateral ovariectomy or no menses during the last 12 months were categorized as postmenopausal. Women reporting a previous hysterectomy and those with missing data were considered premenopausal if they were less than 42 years and postmenopausal if they were more than 55 years old. Altogether 179 women who were equivocal for menopausal status were excluded in the analyses stratified by menopausal status. All participants had given a written consent for future blood samples analyses. The ethical review board of the International Agency for Research on Cancer (IARC) and the local institutions approved the study.

Laboratory assays. Fasting and non-fasting blood samples were collected according to a standardized protocol. From each subject, 30 ml. of blood was drawn using 10 ml. Safety

Monovettes (Sartstedt, Nümbrecht, Germany). Filled syringes were kept at 5-10°C, protected from light, and transferred to a local laboratory for further processing and aliquoting. One dry syringe was used to prepare serum. After centrifugation (1550g for 20 minutes), blood fractions (serum, plasma, red cells, buffy coat) were aliquoted in 28 plastic straws of 0.5 ml each (12 plasma, 8 serum, 4 erythrocytes, 4 buffy coat for DNA), which were heat-sealed at the ends and stored under liquid nitrogen (-196°C). The protocol differed slightly for Denmark. We analyzed samples from women who belonged to different EPIC centers or different menopausal subgroups in a total of 49 separate batches.

We performed all hormone assays on never thawed serum sample aliquots at the laboratory of the Nutrition and Hormones Group, at IARC. We measured serum levels of IGF-I and IGFBP-3 by enzyme-linked immunosorbent assays (ELISA) from Diagnostic System Laboratories (DSL, Webster, Texas). IGF-I assays included an acid-ethanol precipitation step to eliminate IGF-I binding proteins to avoid their interference with the IGF-I measurement. The mean intra- and inter-batch coefficient of variation for IGF-1 was 6.2% and 16.2%, respectively. The corresponding figures for IGFBP-3 were 7.2% and 9.7%.

Statistical analyses. We transformed all measurements of IGF-I and IGFBP-3 using the natural logarithm to normalize the distributions. We calculated Pearsons Partial correlations between IGF-I and IGFBP-3 adjusting for age and batch. The women were categorized according to quintiles and deciles of body height, body weight, BMI, waist- and hip circumferences, and WHR defined over the entire study sample. Subsequently, we calculated the geometric means of IGF-I, IGFBP-3 and the IGF-I/IGFBP-3 molar ratio according to these categories, for all women and according to menopausal status. We used F-test to test for differences between means ($p_{association}$), between the variable categories and serum levels of IGF-I, IGFBP-3 and the molar ratio. We tested for trends across categories of variables by

assigning equally spaced scores to the categories and treating the variables as continuous in the regression analysis (p_{trend}).

We performed univariate and multivariate analyses of the relationship between the following factors; age at enrolment in 5-year intervals (<40, 40-44, 45-49, 50-54, 55-59, 60-64, 65+), age at menarche (<12, 12, 13, 14, 15, 16+), number of children (0, 1, 2, 3, 4, 5+), OC-use (former/never), HRT-use (former/never), smoking (current, former, never), and alcohol consumption in grams/day (0, quintiles of consumption) as the independent variables, and IGF-I, IGFBP-3, and the molar IGF-I / IGFBP-3 ratio as the outcome variable. We did the multivariate analyses adjusting for BMI both as a categorical and continuous variable in order to see if this would effect the displayed associations between the selected variables and the hormone measurements.

Each of the following factors were evaluated as a potential confounder of the relation between the anthropometric measures and serum IGF-I levels; age at enrolment (continuous), study center (ordinal), batch number (ordinal), menopausal status (pre/post), age at menarche (ordinal), number of children (ordinal), OC-use (former/never), HRT-use (former/never), smoking (current, former, never), and consumption of alcohol grams/day (ordinal). Except for age, center, and batch, including the above listed potential confounding variables in the models, did not change the results materially. Since the laboratory assays were performed in separate batches for women according to recruitment centers and menopausal subgroup, only adjustment for batch was done to reduce heterogenity due to protocol differences. Thus, we kept only age and laboratory batch in the final models examining the relationship between the anthropometric indices and the 3 measures of hormonal concentrations.

All tests of statistical significance were 2-sided and *p-values* of < 0.05 were considered statistically significant. We performed univariate and multivariate analyses with the general linear model (GLM). All statistical analyses were done according to the procedures in the

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Statistical Analysis System (SAS) software package, version 8 (SAS Institute, Cary, NC, USA).

Results

The women were aged 32 – 77 years, and 37.9 % were premenopausal. Mean age at study entry was 55 years, and mean age at menopause was 49 years old. The overall crude serum levels for IGF-1 was 31.2 nmol/l and for IGFBP-3 126.1 nmol/l (Table 1). The serum levels of IGF-I was positively correlated to IGFBP-3 with a Pearson partial correlation coefficient of 0.45 after adjustment for age and batch ($p < 0.0001$). Among premenopausal women, the mean serum level for IGF-1 was 29.6 (24.3-36.1) nmol/l and among postmenopausal women 29.6 (25.9-33.5) nmol/l ($p = 0.97$) after adjustment for age and batch. The corresponding figures for IGFBP-3 was 120.0 (113.3-127.2) among pre and 120.8(116.3-125.4) nmol/l among postmenopausal women (p = 0.89).

Table 2 shows the association between quintiles of the six anthropometric indices and the geometric mean of the serum level of IGF-1 and IGFBP-3 with the corresponding ratio after adjustment for age and batch. Body weight, BMI, and waist and hip circumference shows a non-linear relationship with serum levels of IGF-I ($p_{association} < 0.05$) . The non-linearity was most evident for BMI ($p_{association} < 0.0001$), with the highest serum levels of IGF-1 in the third quintile of BMI, i.e 24.6-26.6 kg/m^2. Body height was inverse, while body weight, BMI, waist circumference, and WHR were positively associated with IGFBP-3 ($all\ p_{trend} < 0.05$). Body height was positive and the other antropometric indices inversely associated with the IGF-I/IGFBP-3 molar ratio ($p_{trend} < 0.05$). Similar associations were displayed after stratification by menopausal status (data not shown). Figure 1 displays the non-linear relationship between deciles of weight, BMI, waist and hip circumference with the mean levels of IGF-1. The four antropometric indices all have the lower IGF-1 levels in the bottom and top deciles (Figure1a-d).

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Table 3 shows the results from the multivariate analyses, which included all the selected life style variables and also batch and BMI in quintiles. Age was strongly inversely associated with IGF-1 and IGFBP-3 ($p_{trend} < 0.00001$), while alcohol intake was inversely related to IGF-1 ($p_{trend} = 0.03$) and positively related to the binding protein ($p_{trend} = 0.02$). The displayed results did not change materially when we reran the model, including BMI as a continuous instead of a categorical variable.

Discussion

This large cross-sectional study finds a nonlinear relationship between anthropometric indices of adiposity and serum IGF-I levels. This association is most consistent for BMI. The study also shows that age rather than menopausal status are of importance for the IGF-1 and IGFBP-3 levels. Furthermore, our data show an inverse association between body height, and a positive relationship with four of the five anthropometric indices of adiposity and IGFBP-3. Among the other selected variables only alcohol consumption shows a consistent inverse association with IGF-1 and a positive association with IGFBP-3 levels.

Our strongest finding was the non-linear relationship of serum IGF-I levels with BMI. The previous studies which have examined the relationship between BMI and IGF-1 levels are somewhat inconsistent and shows either no (8-16), or a weak inverse (17-23) association with serum levels of IGF-I.

However, we have specifically examined and found evidence of a non-linear relationship also in other study populations (20, 24-26). In a Swedish population of 445 men and 391 women (20) the highest levels of IGF-I were found, in both sexes, at a BMI of about 24-26 kg/m^2, with the lowest hormone levels in the extreme categories of BMI, although the non-linear relationship achieved statistical significance only among the men. In another study population, comprising 620 women from Italy, Sweden and New York, the non-linear

relationship was revealed among the 443 postmenopausal women with the highest IGF-I level among those with a BMI of between 24 and 25 kg/m^2 (24). In a study of 292 British women the BMI corresponding to the highest IGF-I level was between 26.0 and 27.9 kg/m^2, but the sample size was too small to achieve any statistical significant associations (25). The large cross-sectional study by Holmes et al. (1,037 women) also showed the highest mean level of IGF-I at a BMI between 23-24.9 kg/m^2, although no formal tests were made for existence of a non-linear relationship (22). Finally, in the Multiethnic Cohort, women in the second quartile of BMI, i.e. 23.0-25.0 kg/m^2 also had the highest level of IGF-1, with lower levels among women in the extreme categories of BMI (26).

The non linear relationship displayed in this study between body weight, waist and hip circumference and IGF-1 levels, were all weak. However, taken together they do support the notion of a relationship with measures of adiposity and IGF-1 that is not linear. Besides the relationship with IGF-I, our data showed a consistent positive association between body weight, BMI, waist circumference, and WHR and serum levels of IGFBP-3. A similar direct relationship was present for BMI in the large cross-sectional analysis conducted within the Nurses Health Study (22), and in a British cross-sectional study (25), but not in the Multiethnic cohort (26). The result from our study, find that in spite of the non-linear relationship with BMI, at least for a data set this size, the associations between other lifestyle correlates and serum levels of IGF-1 were not influenced by whether we adjusted for BMI as a categorical or continuous variable in the model.

We previously proposed a physiological model, based on a variety of observations, which may explain the non-linear relationship of adiposity with IGF-I (20, 27). This model is centred around the fact that, fasting and non-fasting insulin levels also tend to increase with increasing BMI. Insulin, on the one hand, sensitizes liver (and probably other tissues) to the stimulatory effects of growth hormone on IGF-I synthesis. On the other hand, insulin

increases free circulating IGF-I by down regulating the synthesis of IGF-binding proteins –1 and –2, and increased free IGF-I, in turn, tends to reduce the pituitary secretion of growth hormone – the primary stimulus for hepatic IGF-I synthesis. Our proposed model is that up to a certain level (of about 25-26 kg/m^2) increasing BMI and increasing insulin levels will enhance IGF-I synthesis. With further increases in the BMI, the increasing negative feedback of free IGF-I on growth hormone secretion will gradually predominate and lead to a reduction in total circulating IGF-I levels. The results from the present study do support this hypothesis.

The inverse association between alcohol consumption and IGF-I found in our study has some support from animal studies (28). However, the epidemiological studies are inconsistent (11, 29))(9, 26, 30-34). In our study, women in the most heavy drinking category i.e. approximately one to two glasses of wine daily drove the inverse trend. In another recent EPIC study including 386 women, we also found a significant and borderline significant inverse relationship between alcohol consumption and IGF-I among pre- and postmenopausal women, respectively (Vrieling et al). Two studies comprising Japanese (30) and American (31) men also found an inverse relationship with moderate alcohol intake and IGF-levels, while several other studies did not (9, 26, 32-34). Furthermore, the Rancho Bernardo study, including both men and women, revealed a positive association between alcohol intake and IGF-I levels (11). We found a positive association between alcohol consumption and IGFBP-3. The previously mentioned Japanese study, including 616 men, also found a positive association with IGFBP-3 (30), while an inverse relationship was shown among women in the Multiethnic cohort (26).

Strengths of the present study are its large size, and the careful exclusion of women who were using exogenous hormones at the time of blood donation – a factor that can substantially

influence IGF-I levels (35-38). We also consider as a strength the wide range of antropometric indices and life style correlates we have in our samples.

A limitation of our study is that the results were based on peptide measurements in only one single blood sample. However, data from the Rancho Bernando study showed that for serum IGF-1 there was not much intra-individual variability (11). Another limitation is the slight differences in protocols for both measurements of anthropometric indices and blood sample handling. However, since we have adjusted for batch we feel confidant that most of this heterogeneity is taken care of by this adjustment. The consistency of the associations displayed in our data also supports the overall results from our study.

In summary, our results show that there is a non-linear relationship between BMI and serum levels of IGF-I, and a positive association between several obesity measures and serum levels of IGFBP-3 among healthy pre and postmenopausal women. The non-linear nature of the relationship may explain some of the very complex effects of IGF-I on disease risk. The association between alcohol consumption and IGF-1 and IGFBP3 - levels is interesting in relation to the etiology of both cardiovascular and cancer diseases and warrants further examination.

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Table 1. Selected characteristics: mean (standard deviation) of the study population (N = 2139)*, all study centres combine; The European Prospective Investigation into Cancer and Nutrition (EPIC)

Characteristics	
Age at entry (years)	54.6 (8.6)
IGF-I (nmol/l)	31.2 (10.1)
IGFBP-3 (nmol/l)	125.9 (45.4)
Molar ratio IGF-I/IGFBP-3	0.276 (0.141)
Body Height (cm)	160.4 (6.8)
Body Weight (kg)	67.6 (11.7)
Body Mass Index (kg/m^2)	26.3 (4.6)
Waist (cm)	82.8 (11.1)
Hip (cm)	103.2 (9.3)
Waist-hip ratio	0.80 (0.06)
Age at menarche (years)	13.1 (1.6)
Age at menopause (years)	49.0 (4.8)
Parous (%)	87.1
Previous OC use (%)	45.7
Previous HRT-use (%)	14.2
Ex smokers (%):	24.1
Current smokers (%):	16.7
Alcohol use (g/day)	7.54 (11.17)

* Some variables have fewer women due to missing values

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Table 2. Geometric means (95% confidence interval) of serum levels of IGF-I (N =2134), IGFBP-3 (N = 2045) and the molar ratio IGF-I/IGFBP-3 (N =2040) by quintiles of anthropometric indices among all women adjusted for age and batch; The European Prospective Investigation into Cancer and Nutrition (EPIC)

	IGF-I (nmol/l)	*IGFBP-3 (nmol/l)*	*Ratio IGF-I/IGFBP-3*
Height (cm)			
<154.5	29.0(28.1-29.9)	121.4(118.6-124.4)	0.237(0.230-0.244)
154.5-158.5	29.7(28.9-30.6)	120.7(118.1-123.4)	0.243(0.236-0.249)
158.5-162.0	29.6(28.7-30.5)	119.7(117.0-122.5)	0.247(0.240-0.254)
162.0-166.0	29.8(28.9-30.6)	119.3(116.7-122.0)	0.245(0.239-0.252)
>166.0	29.6(28.8-30.5)	117.4(114.8-120.0)	0.250(0.243-0.257)
$P_{association}/P_{trend}$	*0.71/0.38*	*0.33/0.04*	*0.17/0.02*
Weight (kg)			
<58.0	29.4(28.6-30.3)	117.9(115.3-120.6)	0.247(0.240-0.254)
58.0-63.5	29.3(28.4-30.1)	117.8(115.3-120.4)	0.247(0.241-0.254)
63.5-68.8	30.3(29.5-31.2)	118.9(116.4-121.6)	0.253(0.246-0.260)
68.8-75.6	30.5(29.6-31.4)	121.7(119.0-124.4)	0.247(0.240-0.254)
>75.6	28.3(27.5-29.1)	121.9(119.2-124.7)	0.229(0.223-0.236)
$P_{association}/P_{trend}$	*0.002/0.37*	*0.08/0.01*	*<0.0001/0.001*
BMI (kg/m^2)			
<22.5	28.9(28.1-29.8)	116.5(113.9-119.1)	0.246(0.239-0.252)
22.5-24.6	29.8(28.9-30.6)	117.1(114.6-119.8)	0.252(0.245-0.259)
24.6-26.6	30.7(29.9-31.6)	121.4(118.7-124.1)	0.250(0.243-0.257)
26.6-29.7	30.3(29.5-31.2)	121.3(118.6-124.0)	0.249(0.243-0.256)
>29.7	28.0(27.2-28.9)	122.1(119.4-124.9)	0.227(0.220-0.233)
$P_{association}/P_{trend}$	*<0.0001/0.35*	*0.01/0.001*	*<0.0001/0.0003*
Waist (cm)			
<73	28.9(28.0-29.8)	114.6(111.9-117.4)	0.249(0.242-0.257)
73-79	30.3(29.4-31.1)	117.8(115.3-120.3)	0.255(0.248-0.262)
79-84	30.2(29.3-31.1)	121.3(118.6-124.1)	0.248(0.241-0.255)
84-92	29.9(29.1-30.8)	120.2(117.7-122.8)	0.245(0.239-0.252)
>92	28.5(27.6-29.3)	124.2(121.5-127.1)	0.226(0.220-0.233)
$P_{association}/P_{trend}$	*0.01/0.33*	*<0.0001/<0.0001*	*<0.0001/<0.0001*
Hip (cm)			
<96	29.2(28.4-30.1)	119.4(116.8-122.2)	0.242(0.236-0.249)
96-100	29.9(29.0-30.8)	118.2(115.5-121.0)	0.251(0.244-0.258)
100-104	30.1(29.2-31.0)	118.6(116.0-121.3)	0.251(0.244-0.258)
104-110	30.1(29.3-31.0)	120.2(117.7-122.8)	0.246(0.240-0.253)
>110	28.5(27.7-29.3)	121.4(118.7-124.1)	0.233(0.227-0.239)
$P_{association}/P_{trend}$	*0.04/0.36*	*0.51/0.19*	*0.001/0.03*
WHR			
<0.75	29.3(28.4-30.1)	113.9(111.4-116.5)	0.254(0.247-0.261)
0.75-0.78	29.5(28.7-30.4)	117.7(115.2-120.3)	0.249(0.243-0.256)
0.78-0.81	29.9(29.1-30.8)	119.2(116.6-121.8)	0.248(0.241-0.254)
0.81-0.85	30.0(29.2-30.9)	122.4(119.7-125.2)	0.243(0.236-0.250)
>0.85	29.0(28.1-29.8)	125.0(122.2-127.9)	0.229(0.223-0.236)
$P_{association}/P_{trend}$	*0.37/0.95*	*<0.0001/<0.0001*	*<0.0001/<0.0001*

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Table 3. Adjusted mean levels of IGF-I (N =2134) and IGFBP-3 (N =2045) by selected variables *; The European Prospective Investigation into Cancer and Nutrition (EPIC)

	Category Definition	*Number*	*IGF1 (nmol/l)*	*Number*	*IGFBP3 (nmol/l)*
Age (yrs)	<40	99	38.7	91	123.3
	40-44	212	32.9	201	117.3
	45-49	356	30.6	342	117.6
	50-54	398	29.6	387	119.2
	55-59	476	28.3	450	116.9
	60-64	328	27.12	317	118.3
	65+	265	24.8	257	113.7
$P_{association}/P_{trend}$			*<0.0001/<0.0001*		*0.34/0.17*
Age at menarche	<12	307	30.6	299	121.3
	12	463	30.3	445	120.5
	13	508	29.7	484	116.6
	14	455	29.8	435	117.9
	15	226	29.1	217	116.6
	16+	150	29.8	140	115.4
$P_{association}/P_{trend}$			*0.41/0.07*		*0.06/0.007*
Parity	0	274	29.3	266	115.0
	1	281	30.1	275	117.8
	2	825	30.6	789	119.8
	3	413	29.4	399	115.0
	4	169	29.3	164	119.0
	5+	91	28.8	82	116.1
$P_{association}/P_{trend}$			*0.12/0.41*		*0.03/0.77*
OC use	Never	1148	29.9	1097	117.1
	Former	963	30.2	925	118.1
$P_{association}$			*0.43*		*0.46*
HRT/ERT use	Never	1766	30.2	1684	121.3
	Former	292	30.5	284	119.2
$P_{association}$			*0.64*		*0.29*
Smoking status	Never	1250	31.1	1199	118.9
	Former	511	31.3	493	118.5
	Current	353	30.3	334	119.2
$P_{association}/P_{trend}$			*0.30/0.30*		*0.93/0.92*
Alcohol intake	0	470	31.1	432	117.1
	<0.84	332	30.7	316	118.1
	0.84-3.30	332	31.1	322	116.9
	3.30-8.13	331	31.4	322	118.97
	8.13-15.39	333	30.9	328	120.3
	>15.39	333	29.2	322	121.4
$P_{association}/P_{trend}$			*0.03/0.03*		*0.23/0.02*

*All factors are mutually adjusted for each other, laboratory batch, and BMI in quintiles

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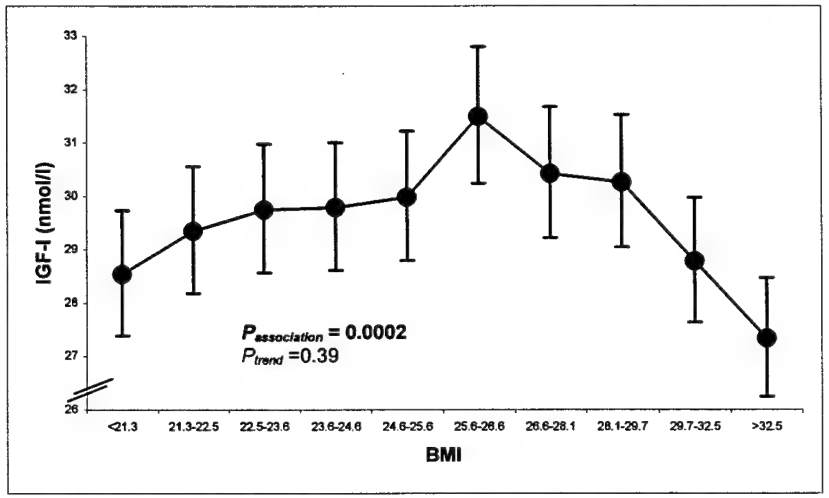
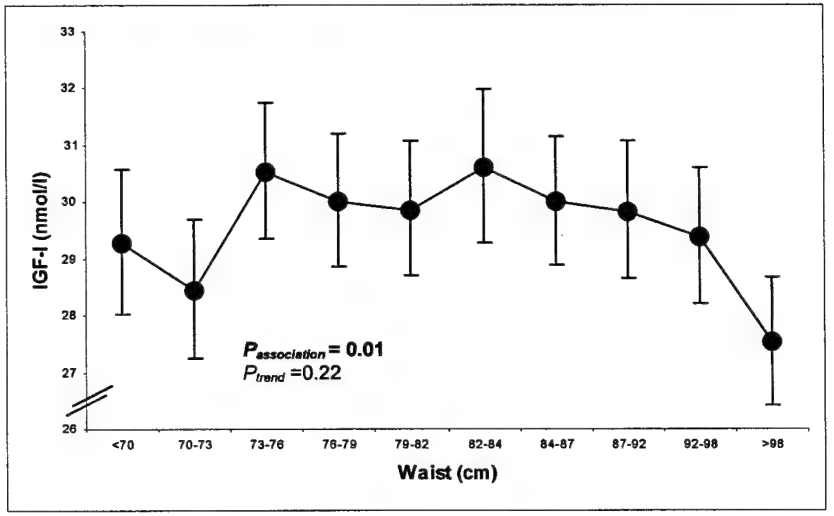
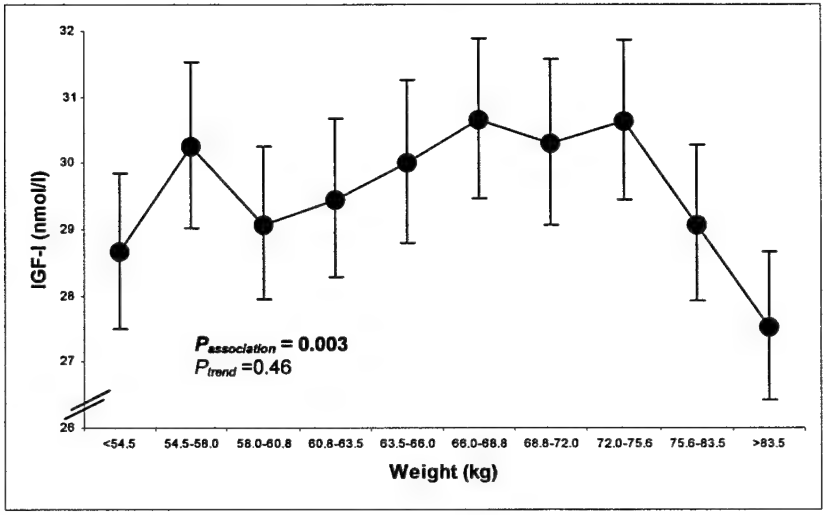
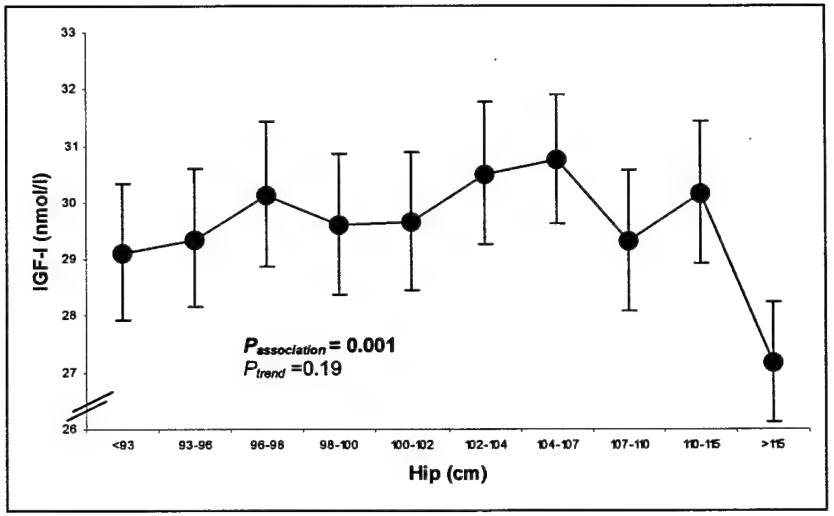
Figure 1a-d. Geometric means (95% confidence interval) of serum samples of IGF-I by deciles of BMI, weight, waist and hip among all women (N = 2139) adjusted for age and batch; The European Prospective Investigation into Cancer and Nutrition (EPIC)









APPENDIX 3

Dietary correlates of insulin-like growth factor (IGF)-I and the IGF binding protein-3 in women.

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Key words: insulin-like growth factor-I, insulin-like growth factor binding protein –3, diet, protein, fruits, milk, dairy products, calcium, alcohol, European Prospective Investigation into Cancer and Nutrition

Running head: Diet, IGF- I and IGFBP- 3

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ABSTRACT

Background: Energy or protein deprivation lower insulin-like growth factor (IGF-I) concentrations. Epidemiologic studies in adequately nourished populations suggest that protein and mineral intake could influence IGF-I and its bioavailability.

Objective: To investigate the dietary correlates of IGF-I and IGF binding protein-3 levels in European women.

Design: Cross-sectional study of the association of diet with serum IGF-I and IGFBP-3 in 2211 European women, who did not use oral contraceptives or hormone replacement therapy at blood donation. Diet was measured through validated questionnaires. Serum hormone concentrations were measured by enzyme-linked immunosorbent assays. Mean hormone levels across categories of dietary variables were calculated by linear regression. Results were adjusted for non-dietary factors found to be associated to IGF-I.

Results: Serum IGF-I concentrations were positively associated with the intake of proteins, dairy products, fruits, dietary calcium and potassium. The increase of mean serum IGF-I in women in the top quintile of intake compared to women in the lowest quintile was 14% for protein, 8% for dairy products, 7% for calcium, 9% for potassium and 5% for fruits. IGFBP-3 was not related with diet. IGF-I was higher and IGFBP3 lower in women consuming more than 12 g of ethanol per day than in other women

Conclusions: Among macronutrients, protein intake plays the most important role in influence in circulating IGF-I in healthy women. Milk, dietary calcium and potassium were positive correlated with IGF-I, together with fruits, while IGFBP-3 was not related to diet. Alcohol intake appears to be a lifestyle determinant of IGF-I and IGFBP-3.

APPENDIX 3

INTRODUCTION

Insulin-like growth factor (IGF)-I is a major endocrine and paracrine regulator of tissue growth and metabolism. Recent studies suggest that higher IGF-I bioactivity may be related to an increased risk of several cancer types (1), including prostate (2), colorectal (3) and premenopausal breast cancer (4). There is also some evidence suggesting that insulin and IGF-I regulate normal cardiovascular physiological responses (5) and that low IGF-I concentrations (and high IGFBP-3) may be associated to the risk of coronary heart disease (6) and the development of type 2 diabetes (7).

Heritability studies have indicated that, at least in well-nourished populations, genetic predisposition factors may determine a large proportion of between-person variation in blood IGF-I concentrations (8;9). Blood levels of IGF-I are dependent on growth hormone and show little variation with sex (10). IGF-I levels are low at birth, increases gradually until puberty, after which the concentration declines slowly with age (11).

Nutritional status is a primary regulator of circulating IGF-I. Energy and/or protein deprivation markedly lower IGF-I concentrations (12). States of chronic starvation such as seen in anorexia nervosa are associated with an increase in growth hormone secretion with a decrease in IGF-I levels (13). Excess energy intake may increase IGF-I somewhat but this does not appear to affect IGF-I levels as strongly as nutritional restriction (14). Little is known about how the diet of adequately nourished individuals affects circulating IGF-I levels. The results from epidemiologic studies suggest that energy balance, together with protein and mineral intake may influence IGF-I levels and its bioavailability (15-21). Regarding food groups, some large studies have reported direct associations of circulating IGF-I with the intake of milk and other dairy products (16;19;22).

The observed relationships of circulating IGF-I levels with the risk of some cancers and other chronic diseases indicate the necessity to better understand the dietary determinants

of IGF-I bioactivity in the population, particularly as such determinants are modifiable. Here, we present the cross-sectional relationship of serum concentrations of IGF-I and its main binding protein IGFBP-3 with dietary factors, in a large sample of 2211 women from ten European countries with very diverse dietary practices (23), participating in the European Prospective Investigation into Cancer and Nutrition (EPIC).

SUBJECTS AND METHODS

The European Prospective Investigation into Cancer and Nutrition

EPIC is an ongoing prospective cohort study designed to investigate the relationships between diet, lifestyle and environmental factors, with the incidence of different forms of cancer. The methods have been described in detail elsewhere (24). EPIC currently includes 519 978 participants (366 521 women and 153 457 men) mostly aged 35-70 years recruited between 1992 and 1998 in 23 centers located in 10 European countries: Denmark (Aarhus, Copenhagen) France, Germany (Heidelberg, Postdam), Greece, Italy (Florence, Naples, Ragusa, Turin, Varese), The Netherlands (Bilthoven, Utrecht), Norway, Spain (Asturias, Granada, Murcia, Navarra, San Sebastian), Sweden (Malmö, Umeå) and the UK (Cambridge, Oxford). The study subjects were recruited from the general population residing in defined geographic areas in each country, except for some cohorts (women of the health insurance for state school employees in France, women attending breast cancer screening in Utrecht, blood donors and their spouses in Ragusa and Spain, and most of the Oxford "health conscious" cohort, which included a large number of vegetarians). Eligible subjects were invited to participate in the study, and those who accepted gave informed consent and completed questionnaires on their diet, lifestyle and medical history. Lifestyle questionnaires included detailed questions about menstrual and reproductive history, current and past use of oral contraceptives, lifetime history of tobacco smoking and consumption of alcoholic beverages, and physical activity. Questionnaires were standardized across all countries contributing to the

present study. Height and weight were measured in most participants while women visited a recruitment center (in the cohort from Malmö and in part of the Oxford cohort, height and weight were self-reported) and blood samples were collected according to a standardized protocol. All participants had given their written consent for future analysis of their blood samples. Approval for this study was also obtained from the ethical review boards of the International Agency for Research on Cancer (IARC) and all local institutions where women had been recruited for the EPIC study.

Diet questionnaires.

Diet over the previous 12 months was measured by country-specific questionnaires to capture local dietary habits and to provide high compliance (25). Most countries adopted an extensive self-administered dietary questionnaire, which can provide data on up to 300-350 food items per country. In Greece, Spain and Ragusa (Italy) the dietary questionnaire was administered by direct interview. A food frequency questionnaire and a seven-day record were adopted in UK, but only data from the food frequency questionnaire are reported here.

A second dietary measurement was taken from an 8% random sample of the cohort, approximately 36,000 subjects, using a computerized 24-hour diet recall method developed *ad hoc* (26). The aim was to calibrate dietary measurements across countries and to correct for systematic over- or under-estimation of dietary intakes.

For this cross-sectional analysis, we investigated the dietary intake of total energy, total protein, total carbohydrate, total fat, saturated, polyunsaturated and monounsaturated fat, cholesterol, alcohol and fiber, and the minerals and vitamins for which the information is available for most of the cohorts: calcium, potassium, vitamin C, beta-carotene and vitamin E. The food groups investigated were meat (red meat, processed meat and poultry), red meat (beef, veal, pork and lamb), processed meat (ham, bacon, sausages and other processed meats from pork and beef), poultry, fish and shellfish, eggs, vegetables, fruits, legumes, potatoes,

cereals, milk, yogurt, cheese, butter, margarines, vegetable oil, sugar and confectionery, cakes and non alcoholic beverages. We excluded 38 women with extreme energy intake/energy requirement ratio (27).

Population of the cross-sectional study.

The women in this cross-sectional study were selected among the control subjects of a case-control study nested within EPIC, on the relationship of breast cancer risk with pre-diagnostic blood levels of endogenous hormones (28). The study included breast cancer cases occurring after blood collection, and matched control subjects, from 19 of the 23 recruitment centers, in eight of the ten participating countries: Denmark, France, Germany, Greece, Italy, The Netherlands, Spain and United Kingdom. Norway was not included in the present study because blood samples have been collected only recently on a sub-sample of cohort participants, and only very few cases of breast cancer had accumulated after blood donation. Sweden was excluded because of limited questionnaire information about menopausal status, past and current oral contraceptive use, and phase of menstrual cycle at the time of blood donation. The study included only women who were free of cancer and did not use oral contraceptives or any hormone replacement therapy at time of blood donation. Fourteen women taking insulin as a medication for diabetes were excluded.

Laboratory assays

In the eight countries contributing to the present study, 30 ml or larger blood samples were drawn, kept at 5-10°C and protected from light, and transferred to a local laboratory for further processing and aliquoting, except in Oxford, UK, where samples were sent to a central laboratory by post at ambient temperature. In seven of the eight countries included in the present study (all except Denmark) blood fractions (serum, plasma, red cells, buffy coat) were aliquoted in 28 plastic straws of 0.5 ml each (12 plasma, 8 serum, 4 erythrocytes, 4 buffy coat for DNA), which were heat-sealed and stored under liquid nitrogen (-196°C). In Denmark, a

non-fasting blood sample was drawn, and aliquoted into serum, plasma, red cells and buffy coats in 1 ml tubes. All samples were processed and frozen within 2 hours at minus 20°C. At the end of the day, all samples were stored in liquid nitrogen vapor (max -150°C). One mirror half of 14 aliquots were stored locally, and the other half centrally at the International Agency for Research on Cancer (IARC), Lyon, France.

All hormone assays were performed at the laboratory of the Hormones and Cancer, at IARC. IGF-I and IGFBP-3 were measured by enzyme-linked immunosorbent assays (ELISA) from Diagnostic System Laboratories (DSL, Webster, Texas). IGF-I assays included an acid-ethanol precipitation step to eliminate IGF-I binding proteins, to avoid their interference with the IGF-I measurement. Measurements were performed on never thawed serum sample aliquots. The mean intra-batch and inter-batch coefficients of variation were 6.2% and 16.2% respectively for IGF-I, and 7.2% and 9.7% respectively for IGFBP-3.

Statistical analysis

In all statistical analyses, measurements of IGF-I and IGFBP-3 were transformed using the natural logarithm to normalize their distributions. The women were categorized according to quintiles of intake of nutrients and foods defined over the entire study sample and geometric means of IGF-I and IGFBP-3 were calculated according to these categories for each of the nutrients and foods. F-tests were used to test for non-linear, overall association between categories of nutrients and foods and serum concentrations of IGF-I and IGFBP-3. Significance levels of linear trends were also based on F-test, by assigning ordinal scores to each successive category and treating the variables as continuous in the regression model.

In all models, energy intake was included for making isoenergetic comparisons, together with age (less than 40, 40-44, 45-49,50-54,55-59,60-64, more than 65 years) and BMI (less than 22.5; 22.5 to 24.6; 24.7 to 26.6, 26.7 to 29.7 and more than 29.7 kg/m^2) as covariates. Analyses were stratified by center to control for differences in questionnaire

design and other center effects. Indicator terms for laboratory batch as a random effect were used in all models as covariates. The inclusion of the following factors as potential confounders did not substantially modify the results and were therefore not included in the final models: alcohol intake (g/day), smoking (current, former, never), menopausal status (pre/post), oral contraceptive use (former/never), hormone replacement therapy (former/never) and work related physical activity (no activity, sedentary, standing, manual and heavy manual activity). We investigated the association of IGF-I and IGFBP-3 with dietary factors in each cohort separately, for the dietary factors for which a significant association in the entire study population was observed.

The analyses were performed with the general linear model (GLM) using the Statistical Analysis System (SAS) software package, version 8 (SAS Institute, Cary, NC, USA). Tests of statistical significance were two-sided. P-values <0.05 were considered statistically significant.

RESULTS

Table 1 shows baseline characteristics of the study population by participating center. Overall, the mean age was 54.5 years (standard deviation, 8.2) and the mean body mass index, 26.2 kg/m^2 (standard deviation, 4.6). The percent of the variance explained by center was 3.9% for IGF-I and 12.5 for IGFBP-3. It was higher for IGFBP-3 compared to IGF-I because the measurements of IGFBP-3 were conducted in different times for some centers, although all in the same laboratory and all using the same method.

We first examined the intake of macronutrients in relation to serum IGF-I concentrations **(Table 2)**. Total energy intake was not associated with serum IGF-I. When we excluded energy from alcohol, mean IGF-I tended to increase with energy intake, with a ~4% increase from the lowest to the top quintile of energy intake (excluding energy from alcohol). The association, although close, was not significant (P_{trend}, 0.06). Serum IGF-I was not

associated with the intake of carbohydrate, total fat, saturated, polyunsaturated, monounsaturated fats or cholesterol, but was directly and significantly related with protein intake. Mean IGF-I values were ~14% higher in women in the highest quintile of protein, as compared to women in the lowest quintile. Dietary fiber was not associated with serum IGF-I.

From the vitamins and minerals we analyzed (Table 2), IGF-I was directly associated with dietary calcium (~7% increase of serum IGF-I when comparing women in the top quintile of calcium intake with those in the lowest quintile) and potassium (~9% increase of IGF-I when comparing women in the top quintile of potassium intake with those in the lowest quintile) but not with the dietary intake of vitamin C, E and beta-carotene.

Next, we analyzed food groups. Fruits and dairy products were the only foods significantly associated with serum IGF-I concentrations **(Table 3)**.

Mean IGF-I was ~8% higher in women in the top quintile of intake of dairy products compared to women in the lowest quintile. The association was significant also for the intake of milk, which is the dairy food most consumed in this population, but not for yogurt or cheese. Dairy products are an important source of proteins (Spearman correlation coefficient (r) adjusted for age, BMI, energy intake and center $r= 0.31$, $P<0.001$) and the most important source of calcium in this population ($r= 0.74$, $P<0.001$). After adjustment for protein intake, the increase of mean serum IGF-I was attenuated from ~8% before adjustment to a ~6% increase of serum IGF-I from the low to the top quintile of dairy products, but the linear trend remained significant ($P_{trend}=0.002$). However, when dietary calcium was included in the regression model, the association of serum IGF-I with the intake of dairy products did not persist. The intake of other foods rich in proteins was not associated with serum IGF-I: meats, red and processed meats, fish and eggs. The relationship with poultry intake showed a positive trend, close to statistical significance (P_{trend}, 0.06).

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The mean IGF-I serum value was ~5% higher in women in the top quintile of intake of fruits compared with women in the lowest quintile of fruit intake. Since dietary potassium was correlated with fruit intake (Spearman correlation coefficient adjusted for age, BMI, energy intake and center, r=0.46, P<0.001) and both potassium and fruit intakes were positively associated with IGF-I, we included potassium as covariate in the regression model. The association was attenuated and the inverse trend was no longer significant (P_{trend} =0.17). Fruits and vegetables combined, vegetables, legumes, potatoes and other tubers, cereals and cereal products, sugar and confectionery, cakes, non-alcoholic beverages, vegetable oil and margarine were not related with serum IGF-I. Butter was significantly associated with IGF-I. The association persisted after further adjustment for intake of dairy products (P_{trend} =0.01).

We examined in each cohort the consistency of the relationship of serum IGF-I with the intake of protein, fruits and dairy products. Mean serum IGF-I values increased from the lowest to the top quartile of intake of dairy products in all cohorts **(Table 4).** For protein intake, the trend of IGF-I to increase was observed in all but one cohort, and for fruits, in 6 out of 9 cohorts.

Serum IGFBP-3 was not related with most of the dietary factors. A significant association was observed only with cheese intake (positive) and processed meat (inverse).

Alcohol intake was inversely associated with serum IGF-I and positively with IGFBP-3. The decrease of serum IGF-I and the increase of IGFB-3 were observed for intakes over 12 g/day of ethanol. The mean of serum IGF-I was ~6% lower in women with daily alcohol intake of 12 or more g of ethanol compared with women with lower intakes of alcohol or with non- consumers. The association with IGF-I persisted after adjustment for IGFBP-3 (P_{trend} =0.0002).

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DISCUSSION

In this cross-sectional study in a population of European women with heterogeneous dietary habits, serum IGF-I concentrations were positively associated with the intake of protein, fruits, dairy products, milk, calcium and potassium (Figure 1) and inversely associated with alcohol intake.

IGFBP-3 was inversely associated with alcohol intake but not with most dietary factors, with the exception of cheese and processed meat. No association was observed with other types of meat or with dairy products. It is therefore possible that the observed associations arose as a consequence of multiple comparisons.

This study is limited by its cross-sectional nature, although it is unlikely that IGF-I levels could influence dietary intakes and food preferences. Our analyses were based on a single measurement of IGF-I and IGFBP-3, but previous data suggest that the intradividual variation in IGF-I and IGFBP-3 is small and that a single measurement may be adequate to represent long-term circulating levels (29). Strengths of the present study are its large size, the inclusion of women from eight European countries with diverse dietary practices and the exclusion of women who were using exogenous hormones at the time of blood donation – a factor that can substantially influence IGF-I levels (30).

Dietary intake of essential amino acids is known to stimulate IGF-I production (14). Our results support than in populations where energy restriction is not prevalent, the role of proteins in influencing IGF-I is more important than that of other macronutrients. Our finding is consistent with the results of earlier large cross-sectional studies in women (19), men (22), and in populations of female vegans and vegetarians (18), and with some intervention trials (12;31;32). No association of IGF-I with protein intake was found in a large multiethnic American population (33) and in several studies with a smaller number of participants (15;17;20;21;34).

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The association of IGF-I levels with the intake of milk and dairy products observed in this study confirms the findings of previous trials (35;36) and large cross-sectional studies (16;19;20). In our study, the association of dairy products with IGF-I was slightly attenuated but persisted after adjustment for protein intake, suggesting that protein could explain only partially the association. Dairy products are rich in calcium, vitamin D, vitamin A and retinol that have been found to be determinants of IGF-I (19) and we observed a positive association of IGF-I with dietary calcium (16;20;32). The association of serum IGF-I with dairy products disappeared after adjustment for calcium in this study. Thus, calcium could be one of the milk components responsible of the potential influence of dairy products on blood IGF-I levels.

It has been argued that bovine IGF-I in cows milk, structurally identical to human IGF-I (37), could lead to increased levels of IGF-I in high consumers of dairy products, particularly in areas where IGF-I in milk is increased by treatment of the cows with recombinant bovine growth hormones. The treatment of cows with growth hormones has not been accepted by the European Commission (38) and therefore this is not a factor in this study population. Some evidence indicate that neonates absorb IGF-I from breast milk (39) but there is no strong evidence that bovine IGF-I in cows milk could be absorbed intact by adults, because it undergoes rapid proteolysis in the upper gut (37;40).

We observed an inverse association of alcohol intake with serum IGF-I, and a direct association with IGFBP-3. Earlier results on the association of alcohol and blood IGF-I have not been consistent (3;15;19;21;29;33), probably because of differences in range of intake between the studies. In the Nurses'Health Study (19) there was a non-linear association between alcohol intake and IGF-I that was somewhat positive for low intakes, but inverse for the highest category of intake. In our study, the decrease of mean IGF-I was observed only for daily ethanol intakes over 12 grams, which is equivalent to a glass of wine per day.

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In conclusion, our results suggest that in well-nourished healthy women serum IGF-I concentrations may be influenced by diet. Protein and dietary calcium intake, and the intake of milk are the strongest nutritional determinants of serum IGF-I in this population **(Figure 1).** Alcohol intake is a lifestyle determinant of both IGF-I (inverse) and IGFBP-3 (positive) concentrations. The evidences on the association of diet and IGF-I with the risk of cancers of the prostate, colon and breast are apparently contradictory. A positive association between dairy products intake and prostate cancer risk has been observed (16), while the association observed with colorectal cancer is inverse (41) and no association with breast cancer risks has been documented (42). Breast cancer risk is increased by consumption of alcohol (43;44), but alcohol intake is not a strong contributor of prostate (45) and colon cancer risk (46). Whether dietary modifications could influence the risk of some diseases through changes in IGF-I is still unclear.

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was in the charge of the laboratory analysis. Laure Dossus performed the statistical analysis. Teresa Norat wrote the report, taking into account the comments and suggestions of the co-authors.

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Table 1. Age, body mass index (BMI), intake of proteins, dairy foods, fruits and alcohol and serum levels of insulin-like growth factor (IGF)- I and the binding protein (IGFBP)-3 in 2211 women by centre. European Prospective Investigation into Nutrition and Cancer

Centre	Number	Age (years)	BMI (kg/m^2)	IGF-I (μg/L)	IGFBP-3 (μg/L)	Proteins (g/day)	Dairy foods (g/day)	Fruits (g/day)	Alcohol (g/day)
Denmark	59	52.3 (1.4)	25.1 (3.7)	316 (290-343)	4973 (4524-5467)	81 (77-84)	254 (207-312)	138 (111-171)	6 (4-9)
France	132	56.0 (8.0)	24.1 (3.6)	249 (236-263)	4918 (4623-5232)	80 (78-83)	232 (203-265)	224 (194-258)	5 (4-7)
Heidelberg (Germany)	54	55.9 (8.4)	26.4 (4.1)	227 (209-247)	2588 (2354-2845)	69 (66-71)	245 (200-301)	118 (96-146)	8 (5-12)
Potsdam (Germany)	63	57.0 (8.7)	27.3 (5.4)	229 (212-247)	3123 (2861-3410)	69 (66-71)	248 (206-300)	143 (117-174)	4 (3-5)
Greece	72	55.5 (10.1)	30.5 (5.4)	213 (198-229)	3333 (3063-3627)	66 (64-68)	136 (114-162)	485 (404-584)	2 (1-3)
Italy	553	52.3 (7.7)	25.5 (4.4)	228 (221-234)	3334 (3234-3438)	79 (78-80)	173 (161-184)	279 (260-299)	3 (3-3)
Spain	376	49.8 (7.7)	28.1 (4.5)	221 (214-228)	3165 (3045-3290)	85 (83-86)	266 (246-289)	251 (231-274)	5 (4-6)
Bilthoven (Netherlands)	65	48.4 (8.2)	25.7 (4.8)	252 (234-272)	4216 (3844-4624)	70 (67-72)	227 (189-273)	151 (124-183)	3 (2-4)
Utrecht (Netherlands)	429	59.2 (6.0)	26.2 (4.3)	240 (232-248)	3159 (3044-3278)	75 (74-77)	408 (377-442)	195 (179-211)	4 (4-5)
Norfolk (UK)	217	61.6 (8.7)	26.3 (4.4)	240 (230-251)	3191 (3037-3353)	80 (79-82)	363 (326-405)	201 (179-224)	4 (3-5)
Oxford (UK)	87	51.3 (7.2)	25.3 (4.3)	226 (212-242)	3314 (3075-3572)	80 (78-83)	351 (299-413)	177 (150-210)	3 (2-4)

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Centre	Number	Age (years)	BMI (kg/m^2)	IGF-I (μg/L)	IGFBP-3 (μg/L)	Proteins (g/day)	Dairy foods (g/day)	Fruits (g/day)	Alcohol (g/day)
Oxford Health Conscious (UK)	104	51.0 (10.3)	23.5 (3.4)	216 (204-230)	3425 (3198-3668)	72 (70-74)	288 (249-334)	250 (214-291)	3 (2-4)

Age and BMI are arithmetic means (standard deviations); IGF-I and IGFBP-3 are geometric means (95% confidence interval) adjusted for age, and BMI; IGF-1 was determined in 2206 women (1 missing subject in France, Heidelberg and Italy, and 2 missing subjects in Spain). IGFBP-3 was determined in 2115 women (2 missing subjects in Heidelberg, Postdam, Norfolk, Oxford, 6 in Greece, 9 in Italy, 42 in Spain, 10 in Bilthoven, 17 in Utrecht and 4 in the Oxford Health Conscious cohort), proteins, dairy foods, fruits and alcohol are geometric means (95% confidence interval) adjusted for energy intake, age and BMI.

APPENDIX 3

Table 2. Mean serum concentrations of IGF-I and IGFBP-3 by daily intake of nutrients (quintiles)

Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
Total energy intake (kcal/day)		
<1539	235	3450
1539-1800	236	3477
1800-2068	242	3441
2068-2407	241	3456
>2407	239	3457
$P_{trend}/P_{heterogeneity}$	0.25/0.51	0.96/0.97
Energy intake excluding calories from alcohol (kcal/day)		
<1477	232	3456
1477-1746	237	3476
1746-2017	242	3434
2017-2333	240	3462
>2333	242	3454
$P_{trend}/P_{heterogeneity}$	0.06/0.24	0.89/0.96
Protein (g/day)		
<61.5	226	3459
61.5-73.7	238	3435

APPENDIX 3

Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
73.7-84.8	236	3394
84.8-98.6	242	3463
>98.6	257	3546
$P_{trend}/P_{heterogeneity}$	**<0.001/0.002**	0.28/0.14
Carbohydrate (g/day)		
<169.8	238	3479
169.8-206.1	240	3483
206.1-243.2	237	3471
243.2-288.0	240	3417
>288.0	237	3434
$P_{trend}/P_{heterogeneity}$	0.96/0.97	0.42/0.85
Total Fat (g/day)		
<56.9	251	3516
56.9-70.1	235	3343
70.1-82.4	240	3437
82.4-98.9	240	3506
>98.9	229	3482
$P_{trend}/P_{heterogeneity}$	0.11/**0.02**	0.43/**0.01**
Saturated Fat (g/day)		
<19.3	245	3458
19.3-24.7	236	3444

APPENDIX 3

Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
24.7-29.8	233	3404
29.8-36.6	240	3456
>36.6	238	3517
$P_{trend}/P_{heterogeneity}$	0.67/0.21	0.48/0.41
Monounsaturated Fat (g/day)		
<20.2	248	3543
20.2-26.0	237	3409
26.0-31.9	236	3420
31.9-41.1	235	3448
>41.1	232	3441
$P_{trend}/P_{heterogeneity}$	0.09/0.22	0.55/0.20
Polyunsaturated Fat (g/day)		
<8.1	239	3453
8.1-10.3	247	3483
10.3-12.8	239	3404
12.8-16.7	234	3483
>16.7	236	3456
$P_{trend}/P_{heterogeneity}$	0.15/0.11	0.98/0.56
Cholesterol		
<180.1	240	3524
180.1-238.7	236	3389

APPENDIX 3

Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
238.7-303.5	236	3411
303.5-387.3	240	3487
>387.3	241	3477
$P_{trend}/P_{heterogeneity}$	0.82/0.76	0.96/0.07
Fiber (g/day)		
<16.9	233	3492
16.9-20.7	240	3454
20.7-23.9	237	3439
23.9-28.4	238	3490
>28.4	245	3403
$P_{trend}/P_{heterogeneity}$	0.11/0.31	0.40/0.53
Alcohol (g/day)		
Non consumers	244	3409
<1.29	238	3453
1.29-5.32	244	3427
5.32-12.62	240	3456
>12.62	228	3533
$P_{trend}/P_{heterogeneity}$	**0.01/0.01**	**0.04**/0.21
Potassium		
<2769	234	3545
2769-3226	245	3561

APPENDIX 3

Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
3226-3653	246	3568
3653-4175	243	3466
>4175	256	3546
$P_{trend}/P_{heterogeneity}$	**0.03/0.05**	0.57/0.51
Calcium		
<731.6	232	3603
731.6-907.7	232	3543
907.7-1081.0	245	3627
1081.0-1233.4	251	3665
>1233.4	249	3647
$P_{trend}/P_{heterogeneity}$	**0.004/0.03**	0.30/0.57
Vitamin C		
<82.0	237	3502
82.0-111.8	236	3455
111.8-144.2	242	3398
144.2	240	3512
>144.2	239	3408
$P_{trend}/P_{heterogeneity}$	0.38/0.69	0.35/0.11
Beta-carotene		
<1318	248	3521
1318-1875	233	3405

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Nutrient	IGF-I (μg/L)	IGFBP-3 (μg/L)
1875-2687	242	3457
2687-3962	237	3476
>3962	236	3438
$P_{trend}/P_{heterogeneity}$	0.17/**0.04**	0.30/0.53
Vitamin E		
<6.6	239	3422
6.6-8.3	240	3456
8.3-10.5	238	3450
10.5-13.6	241	3472
>13.6	235	3473
$P_{trend}/P_{heterogeneity}$	0.69/0.80	0.48/0.95

Adjusted for total energy intake (excluding energy from alcohol), age, BMI, study center and laboratory batch (except the analyses of intake of total energy and energy excluding that from alcohol which were adjusted for the same variables except total energy intake)

APPENDIX 3

Table 3. Mean serum concentrations of IGF-I and IGFBP-3 by intake of foods (quintiles)

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Meat		
<503	243	3465
50.3-75.2	249	3521
75.2-100.3	238	3413
100.3-128.5	240	3489
>128.5	233	3414
$P_{trend}/P_{heterogeneity}$	0.67/0.84	0.31/0.44
Red meat		
<17.5	235	3484
17.5-32.9	240	3443
32.9-47.6	239	3435
47.6-68.4	241	3509
>68.4	239	3419
$P_{trend}/P_{heterogeneity}$	0.50/0.84	0.66/0.44
Processed meat		
<7.0	241	3494
7.0-15.1	242	3562
15.1-23.8	240	3483
23.8-37.9	232	3367
>37.9	238	3406
$P_{trend}/P_{heterogeneity}$	0.17/0.22	**0.004/0.01**

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Poultry		
<5.7	234	3442
5.7-12.9	241	3445
12.9-20.1	234	3415
20.1-39.0	242	3511
>39.0	245	3498
$P_{trend}/P_{heterogeneity}$	0.06/0.10	0.19/0.42
Fish and shellfish		
<8.1	226	3383
8.1-17.9	244	3504
17.9-29.3	244	3454
29.3-48.1	243	3483
>48.1	235	3468
$P_{trend}/P_{heterogeneity}$	0.24/**0.001**	0.35/0.28
Eggs and egg products		
<5.7	237	3481
5.7-10.4	240	3419
10.4-17.2	241	3506
17.2-24.5	234	3455
>24.5	240	3422
$P_{trend}/P_{heterogeneity}$	0.63/0.96	0.50/0.42

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Fruits and vegetables		
<277.5	232	3486
277.5-397.6	242	3454
397.6-520.4	239	3471
520.4-685.4	243	3397
>685.4	239	3458
$P_{trend}/P_{heterogeneity}$	0.21/0.38	0.38/0.58
Fruits		
<131.0	230	3481
131.0-219.8	241	3469
219.8-303.6	241	3461
303.6-431.8	243	3440
>431.8	242	3412
$P_{trend}/P_{heterogeneity}$	0.02/0.05	0.21/0.79
Vegetables		
<105.7	244	3473
105.7-150.6	237	3450
150.6-204.5	237	3401
204.5-295.9	241	3472
>295.9	235	3479
$P_{trend}/P_{heterogeneity}$	0.21/0.38	0.91/0.58

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Legumes		
<1.7	235	3392
1.7-6.0	240	3480
6.0-11.6	241	3534
11.6-24.6	238	3452
>24.6	240	3448
$P_{trend}/P_{heterogeneity}$	0.58/0.79	0.47/0.16
Potatoes and other tubers		
<20.7	242	3463
20.7-46.4	245	3511
46.4-71.7	236	3424
71.7-114.6	236	3462
>114.6	237	3446
$P_{trend}/P_{heterogeneity}$	0.23/0.37	0.66/0.63
Cereals and cereals products		
<120.5	240	3457
120.5-161.0	238	3428
161.0-205.3	239	3432
205.3-269.2	238	3497
>269.2	238	3473
$P_{trend}/P_{heterogeneity}$	0.80/0.99	0.50/0.73

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Dairy products		
<158.4	230	3427
158.4-254.8	234	3439
254.8-360.4	236	3436
360.4-501.4	246	3497
>501.4	249	3486
$P_{trend}/P_{heterogeneity}$	<0.001/0.002	0.20/0.67
Milk and milk beverages		
<25.1	227	3445
25.1-149.2	240	3426
149.2-233.1	239	3493
233.1-400	242	3436
>400	248	3494
$P_{trend}/P_{heterogeneity}$	<0.001/0.003	0.40/0.60
Cheese		
<15.7	236	3411
15.7-29.5	235	3439
29.5-45.7	236	3422
45.7-68.7	241	3467
>68.7	244	3543
$P_{trend}/P_{heterogeneity}$	**0.10/0.44**	0.05/0.22

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
Yogurt		
<2.5	230	3414
2.5-32.3	242	3475
32.3-80.7	239	3474
>80.7	239	3444
$P_{trend}/P_{heterogeneity}$	**0.10/0.44**	0.66/0.59
Sugar and confectionery		
<12.3	240	3504
12.3-23.9	241	3477
23.9-37.3	236	3463
37.3-55.7	235	3456
>55.7	240	3394
$P_{trend}/P_{heterogeneity}$	0.64/0.66	0.09/0.48
Cakes		
<9.8	239	3442
9.8-23.1	235	3446
23.1-39.4	234	3433
39.4-68.8	245	3502
>68.8	240	3446
P (trend)	0.32/0.20	0.63/0.72

APPENDIX 3

Intake (g/day)	IGF-I (µg/L)	IGFBP-3 (µg/L)
Non alcoholic beverages		
<175	242	3594
175-396	240	3482
396-1049	236	3462
1049-1458	237	3382
>1458	240	3454
$P_{trend}/P_{heterogeneity}$	0.94/0.87	0.15/0.09
Vegetable oil†		
<1.89	234	3417
1.89-5.25	239	3504
5.25-15.39	232	3429
15.39-27.71	217	3431
>27.71	228	3509
$P_{trend}/P_{heterogeneity}$	0.81/0.13	0.35/0.48
Butter†		
<0.16	240	3514
0.16-2.22	234	3474
>2.22	227	3421
$P_{trend}/P_{heterogeneity}$	0.01/0.03	0.09/0.44
Margarines†		
<0.08	231	3479

APPENDIX 3

Intake (g/day)	IGF-I (μg/L)	IGFBP-3 (μg/L)
0.08-1.71	233	3433
1.71-11.19	231	3481
>11.19	234	3451
$P_{trend}/P_{heterogeneity}$	0.69/0.80	0.74/0.37

Adjusted for total energy intake (excluding energy from alcohol), age, BMI, study center and laboratory batch (except the analyses of intake of total energy and energy excluding that from alcohol which were adjusted for the same variables except total energy intake)

† Analyses based on 2152 women (cohort from Denmark not included because the dietary item was not provided to the common EPIC database)

APPENDIX 3

Table 4. Changes in serum IGF-I (μg/L) for an increment of quartile of intake of proteins, dairy products and fruit by country in women in the cross-sectional study

	Protein		Dairy products		Fruits	
Country	Δ (μg/L)	P_{trend}	Δ (μg/L)	P_{trend}	Δ (μg/L)	P_{trend}
Denmark	17.3	0.67	13.3	**0.03**	8.8	0.39
France	16.8	**0.01**	7.8	0.29	-1.5	0.27
Germany	-2.6	0.67	8.8	0.12	11.6	**0.01**
Greece	2.9	0.50	24.7	0.05	-2.0	0.91
Italy	4.5	0.08	4.9	0.15	1.7	0.77
Spain	4.4	0.31	4.4	0.14	1.8	0.15
The Netherlands	9.1	**0.001**	6.7	**0.04**	7.0	**0.02**
UK	9.5	0.09	10.1	**0.01**	-1.4	0.71
Health conscious	15.5	0.13	5.6	0.55	4.6	0.58

Δ: Changes in serum IGF-I. Adjusted for total energy intake, age, BMI, laboratory batch. Cohorts within countries were grouped together except for the UK, where the health conscious group in Oxford, comprising a large proportion of vegetarians, was kept separate from the general population samples. Increments are calculated as the difference between quartiles of intake expressed as ordinal variable.

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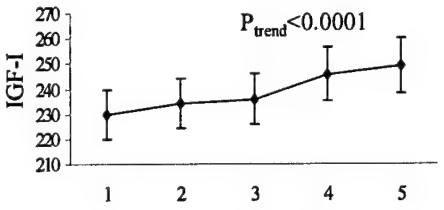
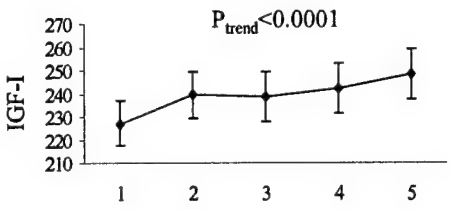
Figure 1. Mean (95% confidence intervals) of IGF-1 concentrations across quintiles of dairy foods, dietary calcium and total protein adjusted for energy intake (excluding energy from alcohol), age, BMI, study center and laboratory batch

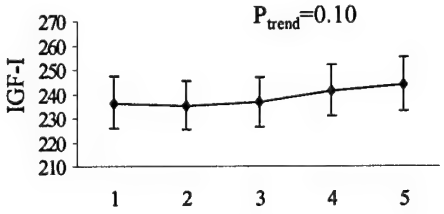
APPENDIX 3



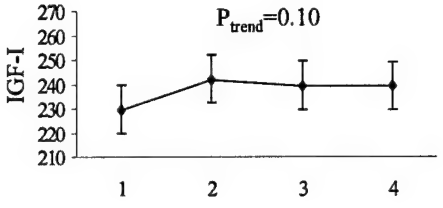
Quintiles of dairy products



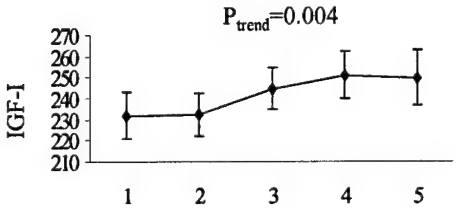
Quintiles of milk



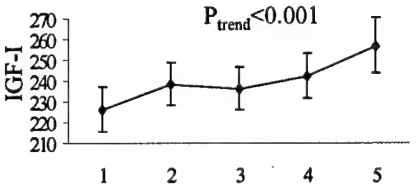
Quintiles of cheese



Quartiles of yogurt



Quintiles of dietary calcium



Quintiles of total protein

APPENDIX 4

Genetic variation in the growth hormone synthesis pathway in relation to circulating IGF-I, IGFBP-3, and breast cancer risk: results from the EPIC study.

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APPENDIX 4

Abstract

Insulin-like growth factor I (IGF-I) stimulates cell proliferation and can enhance the development of tumors in different organs. Epidemiological studies have shown that an elevated level of circulating IGF-I is associated to increased risk of breast cancer, as well as of other cancers. The main stimulus of IGF-I production is growth hormone (GH). Genetic variants affecting the release or biological action of GH may predict circulating levels of IGF-I and have an impact on cancer risk. We tested this hypothesis with a large case-control study of 807 breast cancer patients and 1588 matched control subjects, nested within the European Prospective Investigation into Cancer and Nutrition. We genotyped 25 common single nucleotide polymorphisms in 11 genes involved in GH production and action (*GHRH, GHRHR, SST, SSTR1-5, POU1F1, GH1, GHR*), and, in parallel, we measured serum levels of IGF-I and IGFBP-3, its major binding protein, in samples of cases and controls. *SST* and *SSTR2* polymorphisms showed weak but statistically significant associations with breast cancer risk. *SSTR5* polymorphisms were associated with IGF-I levels, while one polymorphism in *GHRHR* and one in *POU1F1* were associated with IGFBP-3 levels. No polymorphisms were associated at the same time with cancer risk and hormone levels, which suggests that the observed associations for cancer risk are not mediated by alterations in hormone circulating levels. Our conclusion is that common genetic variation in the GH synthesis pathway is not a major determinant of IGF-I and IGFBP-3 circulating levels, and it does not play a major role in altering breast cancer risk.

APPENDIX 4

Introduction

Evidence is rapidly accumulating that insulin-like growth factor-I (IGF-I) can enhance the development of tumors in different organs. Studies *in vitro* have shown that IGF-I inhibits apoptosis and stimulates cell proliferation in a wide variety of cell types (Khandwala *et al.*, 2000). Furthermore, tumor development can be strongly enhanced in animals or organs that have been genetically or otherwise manipulated to either overexpress IGF-I or the IGF-I receptor, whereas animals made deficient in IGF-I are protected (Hadsell *et al.*, 2000). In humans, an increasing number of epidemiological studies have shown increased risks of cancers of the breast (Hankinson *et al.*, 1998; Toniolo *et al.*, 2000, Muti *et al.*, 2002; Yu *et al.*, 2002; Pollak *et al.*, 2004), colon (Ma *et al.*, 1999; Giovannucci *et al.*, 2000; Kaaks *et al.*, 2000; Palmqvist *et al.*, 2002), prostate (Mantzoros *et al.*, 1997; Chan *et al.*, 1998; Stattin *et al.*, 2000; Wolk *et al.*, 1998), and possibly other organs (Petridou *et al.*, 2003), among women and men who have comparatively elevated blood levels of insulin-like growth factor-I (IGF-I), measured either as absolute concentrations or relative to its principal plasmatic binding protein, IGFBP-3. The association of circulating IGF-I levels with breast cancer risk so far has been particularly apparent among women of premenopausal or early menopausal age (Renehan *et al.*, 2004).

Nutrition, especially the availability of energy and amino acids, is a key determinant of circulating IGF-I levels (Thissen *et al.*, 1994; Kaaks & Lukanova 2002). Besides nutrition, however, heritability studies have shown that in western populations a large part (40-60 %) of variation in IGF-I is determined by genetic factors (Hall *et al.*, 1999; Hong *et al.*, 1997; Harrela *et al*, 1996). Although current research to identify genetic determinants of circulating IGF-I and IGFBP-3 is intensifying (Lopez-Bermejo *et al.*, 2000; Jernstrom *et al.*, 2001; Hasegawa *et al.*, 2000; Vaessen *et al.*, 2001), so far few studies have been conducted to search comprehensively for polymorphisms in genes directly or indirectly involved in regulating IGF-I synthesis, and to correlate these with inter-subject variations in IGF-I and IGFBP-3 levels or cancer risk.

The main endocrine stimulus of hepatic and tissue production of IGF-I and IGFBP-3 is growth hormone (GH). Therefore, examination of genetic variants which could affect the pituitary release or biological action of GH may be one way of predicting circulating levels of IGF-I (Lopez-Bermejo *et al.*, 2000). In addition to the gene encoding human growth hormone itself (*GH1*) and its receptor (*GHR*), major candidate genes to be examined are those involved in controlling the pituitary synthesis and release of growth hormone. The latter include growth hormone releasing hormone (*GHRH*) and its receptor (*GHRHR*), as well as somatostatin (*SST*)

and its receptors (*SSTR1-5*), which enhance or inhibit the synthesis and release of GH, respectively. A pituitary-specific transcription factor, called "POU-domain class 1 transcription factor 1" (*POU1F1*) is also centrally involved in regulating GH synthesis.

For each of these genes, polymorphisms that change gene expression or protein function might result in a relative increase or decrease in circulating IGF-I or IGFBP-3 levels. In several of these genes, rare genetic mutations have been identified that result in radically altered hormone levels and in growth related diseases such as acromegaly or familial dwarfism (Pernasetti *et al.*, 1998; Ballare *et al.*, 2001; Salvatori *et al.*, 2001; Wajnrajch *et al.*, 2003). However, only a few studies have shown associations between more common polymorphisms and variation of IGF-I levels compatible with normal physiology (Jernstrom *et al.*, 2001; Vaessen *et al.*, 2001).

To examine whether common genetic variants of *GHRH*, *GHRHR*, *SST*, *SSTRs*, *POU1F1*, *GH1* and *GHR* were associated with variations in circulating IGF-I and IGFBP-3 levels, and possibly also with breast cancer risk, we conducted a large case-control study of 807 breast cancer patients and 1588 matched control subjects, nested within the cohorts of the European Prospective Investigation into Cancer and Nutrition (EPIC) (Riboli *et al.*, 2002; Bingham and Riboli, 2004). For the present study, an attempt was made to include all known, common polymorphisms which have the highest chance of having an impact on gene expression or function of the gene product.

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Material and methods

The EPIC study

The EPIC cohort consists of about 370,000 women and 150,000 men, aged 35-69, recruited between 1992 and 1998 in 23 research centres in 10 Western European countries (Riboli *et al.*, 2002; Bingham & Riboli, 2004). The vast majority (>97%) of subjects recruited in the EPIC cohort are of European ("Caucasian") origin. All EPIC study subjects provided anthropometric measurements (height, weight, waist & hip circumferences), and extensive, standardized questionnaire information about medical history, diet, physical activity, smoking, and other lifestyle factors. Women also answered questions about menstrual and reproductive history, hysterectomy, ovariectomy, and use of exogenous hormones for contraception or treatment of menopausal symptoms. In addition, about 240,000 women and 140,000 men provided a blood sample, and plasma, serum, red cells, and a buffy coat were stored for future analyses on cancer cases and controls. Cohort members are contacted regularly to renew information on smoking, alcohol drinking, physical activity, weight, menstrual history, pregnancies, use of medications and exogenous hormones, hysterectomy, and first development of major diseases other than cancer (e.g. diabetes).

Cases of cancer occurring after recruitment into the cohort are identified through local and national cancer registries in 7 of the 10 countries, and in France, Germany, and Greece by a combination of contacts with national health insurances and/or active follow-up through the study subjects or their next of kin. Follow-up on vital status, to monitor the population remaining at risk for cancer, is achieved through record linkage with mortality registries. In all EPIC study centers, cancer diagnosis is confirmed through comprehensive review of pathology reports, and checks for completeness of follow-up are made regularly. A fully detailed description of the EPIC study has been published elsewhere (Riboli *et al.*, 2002; Bingham and Riboli, 2004).

Selection of case and control subjects.

Cases and controls from the present study were from 16 of the 23 EPIC recruitment centers, in 7 of the 10 countries (U.K., Germany, Netherlands, France, Spain, Italy and Greece), and most were also part of nested case-control studies on serum hormones and breast cancer risk reported in detail elsewhere (Kaaks *et al.*, 2 manuscripts submitted, Rinaldi *et al.*, manuscript in preparation).

Case subjects were selected among women who developed breast cancer after their recruitment into the EPIC study, and before the end of the study period, for each study center

defined by the latest end-date of follow-up. Women who used any hormone replacement therapy at the time of blood donation, or any exogenous hormones for contraception or medical purposes, and who had previous diagnosis of cancer (except non-melanoma skin cancer) were excluded from the study, because each of these various factors could have altered circulating hormone levels.

For each case subject with breast cancer, two control subjects were chosen at random from among cohort members alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. Control subjects were matched to the cases by study center where the subjects were enrolled in the cohort, as well as by menopausal status (pre-menopausal, post-menopausal, peri-menopausal/undefined), age (± 6 months) at enrollment, follow-up time (Kaaks *et al.*, manuscripts submitted), fasting status, time of the day of blood donation, phase of the menstrual cycle for premenopausal women.

Approval for the study was given by the relevant Ethical Committees, both at the IARC and in the EPIC recruitment centers.

Identification and selection of SNPs

We collected data on polymorphisms from publicly available databases, such as dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), SNPper (http://snpper.chip.org/) and Frequency Finder (http://bluegenes.bsd.uchicago.edu/frequencyfinder/). We complemented database searches with literature review and, for some genes (*SST, SSTR1-5, GHRH, GHRHR, POU1F1*) with analysis of 95 subjects from the EPIC population by denaturing high-performance liquid chromatography (DHPLC; Xiao and Oefner, 2001).

To be included in the study, polymorphisms had to be located in exons (including untranslated regions), exon-intron junctions, or promoter regions of a gene of interest, or otherwise should be within intronic regions that showed greater than 80% homology between human and mouse (as reported by the UCSC Genome Browser, http://genome.ucsc.edu/), and thus were likely to harbor regulatory sequences. In addition, we also included polymorphisms with documented evidence of their existence in Caucasians, either according to literature data or to our own experimental analysis by DHPLC. Among all polymorphisms thus identified, we only retained those with a minor allele frequency $\geq$5% in Caucasians, or those that result in an aminoacid change and had a minor allele frequency $\geq$1%. Finally, we particularly favored the inclusion of all polymorphisms previously reported in the literature to be associated with cancer and/or levels of circulating hormones.

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Genotyping

Buffy coat samples for the study subjects were retrieved from the EPIC biorepository and DNAs were extracted on an Autopure instrument (Gentra Systems, Minneapolis, MN) with Puregene chemistry (Gentra Systems, Minneapolis, MN).

Genotyping was performed by the 5' nuclease assay (TaqMan). The order of DNAs from cases and controls was randomized on PCR plates in order to assure that an equal number of cases and controls could be analyzed simultaneously. Taqman probes were synthesized by either Applied Biosystems (with MGB chemistry) or Proligo (with or without LNA chemistry). Sequences of primers and probes are reported in Supplementary Table 1. The reaction mix included 10 ng genomic DNA, 5 pmol of each primer, 1 pmol of each probe, and 2.5 µl of 2 X master mix (Applied Biosystems) in a final volume of 5 µl. The thermocycling included 50 cycles with 30 seconds at 95 °C followed by 60 seconds at 60 °C. PCR plates were read on an ABI PRISM 7900HT instrument (Applied Biosystems). In order to validate genotype identification we repeated 8% of all genotypes. Laboratory personnel was kept blinded to case-control status throughout the study.

Hormone measurements

Measurements of IGF-I and IGFBP-3 were performed in the laboratory of the Hormones and Cancer, at IARC, using enzyme-linked immunosorbent assays (ELISA) from Diagnostic System Laboratories (DSL, Webster, Texas). The IGF-I assays included an acid-ethanol precipitation step to eliminate IGF-I binding proteins, to avoid their interference with the IGF-I measurement. Measurements were performed on never thawed serum sample aliquots. The mean intra-batch and inter-batch coefficients of variation were 6.2% and 16.2% respectively for IGF-I, and 7.2% and 9.7% respectively for IGFBP-3.

Statistical analysis

Inviduals' haplotype frequencies (i.e., estimated numbers of copies of haplotypes) were reconstructed using the program "tagSNPs" (http://www-rcf.usc.edu/~stram/tagSNPs.html) by Stram *et al.* (Stram *et al.*, 2003; Stram *et al.*, 2003). This program calculates, for each individual, the expected numbers of copies ("dosages") of each of the haplotypes compatible with the individuals' SNP genotypes. This method takes account of uncertainties in the haplotype reconstruction for individuals that are heterozygote for 2 or more of the SNPs

within a given gene. Haplotype dosages are estimated from the individuals' SNP genotype data, and from overall haplotype frequency estimates for the full study population (cases and controls combined) estimated by a maximum likelihood method. For each haplotype, the dosage values range from 0 to 2.0 (alleles), and for each individual these dosage values add up to a total value of 2.0 across all possible haplotypes.

All association analyses, at the level of individual SNPs or gene loci, were performed under different assumed modes of inheritance of effect – dominant, recessive, or co-dominant – associated with alleles. In the 'dominant' model, circulating peptide levels or disease risks were compared between subjects carrying at least one copy of the rare allele, and those who had none; in the 'recessive' model, the comparison was between those who were homozygous for the rare allele, and all others; in the 'codominant' model, individuals' peptide levels or the logarithm of disease risk were linearly related to the number of copies of an allele (0, 1, or 2 for SNP alleles, or dosages for the haplotype) carried by the individuals. For rare alleles, with a frequency less than 20% (i.e., a prevalence of homozygous recessive allele carriers less than 4.0%), only the dominant model was used. To test whether any association of gene variants with breast cancer risk could be mediated by alterations in circulating levels of IGF-I and/or IGFBP-3, these associations were also estimated with adjustment for serum peptide levels.

Relationships of polymorphic gene variants with serum levels of IGF-I and IGFBP-3 were estimated by standard normal regression models, stratified by EPIC recruitment center and further adjusted for age. Relationships of polymorphic variants with breast cancer risk (odds ratios [OR]) were estimated using conditional logistic regression models, applied on the matched case-control sets. Both series of analyses were performed at the level of single SNP loci, as well as at the level of haplotypes (using the haplotype dosage values). Haplotype analyses were performed at the level of full gene loci – i.e., including haplotypes based on all of the SNPs in that gene. In all haplotype analyses, the most common haplotype was used as the reference category.

Subgroup analyses on women with a breast cancer diagnosis either before (45% of the subjects), or after age at diagosis of 55 years (the age at which over 99% of women enrolled in the EPIC cohort declared themselves menopausal) were used to examine whether associations of gene variants with breast cancer risk differed between women with cancer an approximately premenopausal or postmenopausal age, and possible heterogeneity of effect between these two age groups was tested using a chi square test.

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Results

Eight hundred seven incident cases of breast cancer from the EPIC cohort and 1588 matched controls were included in our study. The mean age of study subjects was 55 years (5^{th}-95^{th} percentile: 39.9-68.7 years). For cases, the mean age at diagnosis was 57 years (5^{th}-95^{th} percentile: 42-71 years). Based on the questionnaire data, 32% of the subjects were pre-menopausal at blood donation, 10% were peri-menopausal or of unknown menopausal status, and 58% were post-menopausal. Cases had a significantly lower number of full-term pregnancies than controls (means: 2.35 *vs.* 2.47, p=0.02) and were significantly older at first full-term pregnancy (26 years *vs.* 25.5 years in controls, p=0.02). Age at menarchy did not differ between cases and controls, nor did body mass index. Serum levels of IGF-I were not significantly different between cases and controls (means: 242 ng/ml *vs.* 237.5 ng/ml, p=0.3), whereas cases had more elevated levels of serum IGFBP-3 than controls (means: 3016 ng/ml *vs.* 2936 ng/ml, p=0.02). The difference in IGFBP-3 levels was not observed in the subgroup of women younger than 55, and it was entirely confined to the older age group. Details on the relationships of IGF-I and IGFBP-3 with breast cancer risk analyses will be reported elsewhere (Rinaldi *et al.*, in preparation).

We collected information on polymorphisms from the literature, public databases and our own experimental analyses by DHPLC. This provided a list of 77 SNPs. By applying the selection criteria outlined in the Methods section, we selected 35 SNPs for genotyping. For 2 SNPs, genotyping assays could not be designed and for 8 more SNPs, TaqMan assays were generated but provided poor genotyping results. This left 25 polymorphisms that were genotyped on the DNAs of cases and controls (Table 1). The number of SNPs typed per gene ranged from one for the small somatostatin receptors *SSTR1*, *SSTR3*, *SSTR4* to five for *GHRHR*.

Genotyping call rates ranged between 95.36% and 99.53%. The distibutions of genotypes of all polymorphisms were in agreement with Hardy-Weinberg equilibrium. Repeated quality control genotypes (8% of the total) showed greater than 99% concordance for all assays. Results of associations between individual SNPs and cancer risk and circulating hormone levels are reported in Table 2. Tables 3a to 3e report results of analyses of haplotypes of genes for which two or more polymorphisms have been typed.

Serum concentrations of IGF-I showed a nominally significant association only with one SNP (P0827, p=0.01) in *SSTR5*, but not with any polymorphisms in the other genes studied. Haplotype analysis did not add anything to this finding, as there was a significant association with the only *SSTR5* haplotype (hCCCA) which includes the A allele of P0827. When we

stratified the data by age at diagnosis (before or after age 55), the association was observed only in women younger than 55 (data not shown).

Significant associations with reduced levels of IGF-I were also observed for two haplotypes of GH1, namely hTCAT and hTCGG, although no single SNP of this gene was associated with IGF-I level.

Serum IGFBP-3 concentrations were significantly increased among homozygous carriers of the P0353 C allele in *GHRHR* (p=0.03). In addition, carriers of the P0593 C allele of *POU1F1* had significantly elevated levels of IGFBP-3 (p=0.004 and p=0.006 for codominant and dominant model, respectively).

Breast cancer risk showed statistically significant associations ($p<0.05$) with polymorphic variants in the *SST*, *SSTR2*, and *GH1* genes.

For the *SST* gene, carriers of two different SNPs showed an increase in breast cancer risk, with relative risks of about 1.3 for both the P0692 C and P0689 C alleles. Reflecting these two associations, the hCC haplotype of *SST* also showed an effect on risk (OR=1.27, 95% CI=1.02-1.59 for the dominant model). In analyses stratified by age at diagnosis, a statistically significant increase in risk was observed only in the higher age group (OR=1.53, 95% CI=1.17-2.01 for carriers of the P0692 C allele and OR=1.41, 95% CI=1.07-1.88 for carriers of the P0689 C allele), but not among women with a breast cancer diagnosis at age below 55. However, interaction tests showed no statistically significant heterogeneity of effect between the two age groups.

For the *SSTR2* gene, breast cancer risk was decreased among homozygous carriers of the C allele of SNP P0836 (OR=0.74, 95% CI=0.57-0.96). This reduction in risk was mirrored by by an increased risk associated with the haplotypes bearing the other allele (hGG, OR=1.24, 95% CI=1.03-1.51, and hAG, OR=1.19, 95% CI=0.99-1.45). Heterozygosity at the other *SSTR2* polymorphism we typed (P0837) was associated with a nearly significant increase in breast cancer risk (OR=1.20, 95% CI=0.99-1.45).

For the *GH1* gene, only subjects who were heterozygous for the P0323 allele an association with reduced cancer risk (OR=0.77, 95% CI=0.63-0.94), which was compatible however with a dominant effect of the G allele towards a reduction in risk (p=0.03).

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Discussion

We have performed a large-scale association study, nested in the EPIC cohort, to assess the role of genetic variation of genes involved in the growth hormone synthesis pathway on risk of breast cancer and on circulating levels of IGF-I and IGFBP-3. The present study was conducted on a large subset of the breast cancer cases and matched control subjects that were included in a parallel study on breast cancer risk in relation to IGF-I and IGFBP-3 (Rinaldi *et al.*, manuscript in preparation), as well to circulating sex steroids (Kaaks *et al.*, manuscripts submitted). To our knowledge, this is the first study to look in a comprehensive way at breast cancer risk and genetic variation in the growth hormone synthesis pathway – including a total of eleven different genes – and the first study to simultaneously analyze associations of genetic variants with circulating levels of IGF-I and IGFBP-3 in the European population.

Our objective was to include into our analyses all SNPs that would have a minimum allele frequency of 5 percent, or otherwise a high chance of having an impact on gene expression or function of the gene product (e.g., known coding variants). We therefore performed extensive searches through the literature and public databases. Although the DHPLC technique approach may be somewhat less sensitive than a systematic resequencing for the identification of new SNPs, it has been shown to be a quite reliable method for SNP detection (reviewed in Xiao and Oefner, 2001). While systematic resequencing would have probably led to the identification of still further polymorphisms, this approach did not fall within the financial scope of our project. We believe, however, that most of the additional SNPs that could have been identified by such more stringent approach probably will have an allele frequency below 5 percent, and that resequencing would have led to the identification of only very few additonal common polymorphisms, with higher allele frequencies. Overall, therefore, we are confident that we have included in our study most of the common variants existing in Caucasians in the eleven candidate genes examined in this study.

While we have tried to have a fair representation of all common variants, we took great care in retaining for our study only those polymorphisms for which evidence of experimental validation was available. This was particularly relevant for SNPs of the *GH1* gene, given that it belongs to a cluster of five genes which all share very high degree of homology, even for intronic and promoter regions (Chen *et al.*, 1989). Although many polymorphisms have been reported in the *GH1* gene, several of these may have been actually artifacts due to non-specific amplification of target sequences in one of the other *GH* homologues. In this regard, it is important to note that none of polymorphisms we have typed in the *GH1* gene showed

any significant departure from Hardy-Weinberg equilibrium, which suggests that our genotyping assays for *GH1* gene polymorphisms have been properly designed.

Our study population included women from 7 out of the 10 countries participating in the EPIC project, ranging from southern Europe (Greece, Italy, Spain) to the UK. Over 97% of EPIC subjects are estimated to be of Caucasian origin. Nevertheless, there is substantial regional difference in breast cancer incidence rates across Europe (most likely due to differences in lifestyle). Thus, spurious associations of risk with allelic variants could be found if allelic frequencies varied substantially between regions. This potential bias was avoided, however, by matching the controls subjects to the breast cancer cases by EPIC recruitment center, and by performing a conditional logistic regression analysis. Regression models relating IGF-I or IGFBP-3 to polymorphic variants were also systematically adjusted for the factor "recruitment center".

Serum levels of IGF-I and IGFBP-3 showed statistically significant associations with variants (SNPs, haplotypes) in several of the candidate genes studied. However, in spite of the large size of this cross-sectional study component, most of these associations were not highly statistically significant (p-values ranging between 0.02 and 0.05). The only exception was the association between a polymorphism in the promoter of *POU1F1* and IGFBP-3 level, supported by a p-value of 0.004. This novel finding will have to be confirmed by further epidemiological and/or functional studies. Furthermore, for none of the SNPs found to be associated with serum peptide levels was there any previous evidence of a similar association, or any experimental evidence for a possible direct, functional role. This makes it difficult to assess whether the associations observed in our study represent a true effect on serum peptide levels (directly, or through linkage disequilibrium with other, functional polymorphisms), or whether they were merely chance findings. Additional large association studies will be needed to confirm our findings.

With regard to breast cancer risk, the only relatively consistent pattern of associations was with variants of the *SST* and *SSTR2* genes. For none of these variants, however, was there a parallel association in IGF-I or IGFBP-3 levels. This suggests that the observed associations may not be mediated by alterations in circulating and/or tissue levels of IGF-I and/or IGFBP-3. It is, indeed, possible that associations of breast cancer risk with polymorphisms in the *SST* and *SSTR* genes reflect autocrine or paracrine mechanisms of cellular proliferation active at the level of the breast epithelium. Both somatostatin (Nelson *et al.*, 1989) and its receptors (Bootsma *et al.*, 1993) have been found to be produced by breast cancer cells, and all five somatostatin receptors have been found in human breast tumors, with subtype 2 occurring

more frequently (Xu *et al.*, 1996). The presence of somatostatin receptors in human breast cancers has been correlated with well-differentiated tumors and more favorable prognosis (Bootsma *et al.*, 1993), and treatment with somatostatin or its synthetic analogs has been shown to have antiproliferative effects on breast tumors, both *in vitro* and *in vivo* (Mascardo and Sherline, 1982), and to result in a positive tumor response in over 40% of patients (Dolan *et al.*, 2001).

In view of the above observations, it is possible that polymorphisms which affect expression of the somatostatin gene *SST* and/or its receptor *SSTR2* could modulate the antiproliferative effect that somatostatin exerts on breast tumors. The *SST* SNPs that we found to be associated with cancer risk are located in intron 1 of this gene, and nothing is known of their possible function. The association we report may reflect either a direct functional effect of the polymorphisms studied, or may be due to linkage disequilibrium with unknown functional polymorphisms. On the other hand, the *SSTR2* SNPs we have typed are located in the promoter, respectively at positions −57 and −83 upstream of the start of transcription. Interestingly, the *SSTR2* haplotype (hGG) that shows an increased risk of breast cancer has been also reported to be associated with a 60-70% reduction of *SSTR2* transcription in pancreatic cancer cells, by use of site-directed mutagenesis and a luciferase reporter gene assay (Torresani *et al.*, 2001). It has to be noted that, at the individual SNP level, it was the polymorphism at position −83 that was found to be responsible for this decrease (Torrisani *et al.*, 2001). We found a borderline, non-significant association with the SNP at the -83 position, and a significant association with the SNP at −57. In our sample there is complete but not perfect linkage disequilibrium between these two polymorphisms (D'=1, r^2=0.36), reflecting the fact that their frequencies are different (minor allele frequencies of 32% and 44%, respectively). It is difficult therefore to say whether the associations we observe and the previously reported functional role for one of the *SSTR2* promoter SNPs are in relation or not.

Only one SNP included in our work has been previously studied in relation to breast cancer or circulating levels of IGF-I and IGFBP-3. SNP P0320, located in intron 4 of *GH1*, has been found to be associated with level of IGF-I and risk of colorectal neoplasia (Le Marchand *et al.*, 2002). Another study found an association of this polymorphism with secretion of growth hormone, IGF-I and with stature in a group of Japanese prepubertal short children (Hasegawa *et al.*, 2000). The same polymorphism did not show any association with breast cancer risk, however, in a large case-control study performed in a Chinese population (Ren *et al.*, 2004). Likewise, we did not observe any association of this polymorphism with cancer risk or hormone level in our study. Nevertheless, we have found two *GH1* haplotypes

that showed a weak association with reduced IGF-I level. This leaves the possibility that the previously reported associations reflected linkage disequilibrium with other polymorphisms.

Previous prospective cohort studies have shown increased prediagnostic IGF-I levels among women who developed breast cancer, especially when the cancer was diagnosed at a relatively young (premenopausal or early menopausal) age. Contrary to these previous findings, however, our data from the EPIC cohort showed a weak, direct association of serum IGF-I with breast cancer risk only among women of postmenopausal age, but not among the younger women (Rinaldi *et al.*, manuscript in preparation). In the present study, we also performed analyses of genetic variants in relation to breast cancer risk stratified by age at breast cancer diagnosis (below and above age 55). Our study was not large enough, however, to allow for statistically powerful tests for differences in associations between the older and younger women, especially in relation to the rarer polymorphisms.

In summary, We have performed a large study which, for the first time, investigated the role of genetic variation across eleven different genes belonging to the same pathway. This obviously entailed a large number of statistical tests, which may have led to a number of spurious associations due to chance. One approach to account for the multiple comparisons is to use Bonferroni's method, applying a more stringent criterion for statistical significance, at the level of each gene studied. This method is known to be conservative, however, as its is difficult to account for dependence between statistical tests due to linkage disequilibrium between SNPs. Most of the associations observed in this study were relatively weak and not supported by strong p-values. Only the association between a SNP in *POU1F1* and IGFBP-3 levels was supported by a $p<0.01$. An alternative is to apply a Bayesian approach to calculate false positive response probabilities (FPRP) (Wacholder *et al.*, 2004). We have computed FPRPs for the nominally significant associations we have observed between SNPs and breast cancer risk. Use of a prior probability of 0.1 resulted in noteworthy FPRPs for the association with breast cancer risk of polymorphisms P0323 in *GH1* (FPRP=0.16) and P0692 in *SST* (FPRP=0.16). When using a prior probability of true association of 0.01, which is more likely to be correct, we have obtained high FPRP values, ranging from 0.67 to 0.88.

In conclusion, the associations we report here do not have a strong statistical support. Therefore, replication will be key to confirm or dismiss the results of our study. Given that associations with individual genetic variants appear to be of a relatively small magnitude, even larger studies will be needed to confirm our findings, and to allow for associations studies on rarer polymorphic variants as well.

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Table 1. Polymorphisms used in the present study.

Gene	Polymorphism[a]	Alleles (major>minor)	Position in gene	Codon
GHRH	***GHRH***			
P0342 A/G	rs4988491	A>G	Downstream of 3'UTR	-
GHRHR	***GHRHR***			
P0347 C/T	rs4988495	C>T	Intron 2	-
P0348 G/A	rs4988496	G>A	Exon 3	Ala57Thr
P0353 T/C	rs4988501	T>C	Intron 5	-
P0359 C/T	rs4988503	C>T	Intron 10	-
P0360 C/G	rs4988505	C>G	Intron 12	-
GH1	***GH1***			
P0322 G/T	rs2005172	G>T	Promoter	-
P0323 A/G	rs6171	A>G	Promoter	-
P0327 C/A	rs6173	T>G	Exon 1, 5'UTR	-
P0320 T/A	rs2665802	T>A	Intron 4	-
GHR	***GHR***			
P0329 G/A	rs6179	G>A	Exon 6	Gly186Gly
P0332 G/T	rs6182	G>T	Exon 9	Cys440Phe
P0335 T/G	rs6180	T>G	Exon 9	Ilu544Leu
SST	***SST***			
P0692 T/C	rs4988513	T>C	Intron 1	-

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P0689 T/C	P0689[b]	T>C	Intron 1	-
SSTR1	***SSTR1***			
P0694 T/C	rs2228497	T>C	Exon 1	Val293Val
SSTR2	***SSTR2***			
P0837 A/G	rs998571	A>G	Promoter	-
P0836 G/C	rs1466113	G>C	Promoter	-
SSTR3	***SSTR3***			
P0702 G/A	rs229569	G>A	Exon 2	Ala264Ala
SSTR4	***SSTR4***			
P0710 T/G	rs3746726	T>G	Exon 1	Phe284Val
SSTR5	***SSTR5***			
P0719 C/A	rs4988483	C>A	Exon1	Leu48Met
P0723 C/T	rs4988487	C>T	Exon 1	Pro109Ser
P0727 T/C	rs169068	T>C	Exon 1	Leu335Pro
P0827 G/A	rs642249	G>A	Exon 1	Pro348Pro
POU1F1	***POU1F1***			
P0593 C/T	rs300982	C>T	Promoter	-

[column in red will be omitted from the final version of the manuscript. It is left temporarily for ease of consultation]

[a] Polymorphisms are identified by their dbSNP accession number. dbSNP is accessible at http://www.ncbi.nlm.nih.gov/SNP/

[b] P0689 is an internal reference. This polymorphism is not represented in dbSNP. See ref. Naylor *et al.*, 1983

APPENDIX 4

Table 2. Associations between SNPs and breast cancer risk and mean IGF-I and IGFBP-3 levels[a] adjusted for age and center

Gene	SNP		Genotype			$P_{Codominant}$[b]	$P_{Dominant}$[c]	$P_{Recessive}$[d]
			Homozygous major	Heterozygous	Homozygous minor			
GH1	P0320 T/A	Cases/Controls	276/495	350/728	148/278			
		OR (95% CI)[e]	1.00	0.86 (0.70-1.04)	0.95 (0.74-1.22)	0.48	0.18	0.12
		Mean IGF-I [f]	242.6	243.9	248.0	0.21	0.41	0.21
		Mean IGFBP-3 [f]	3515	3515	3580	0.17	0.56	0.07
	P0327 C/A	Cases/Controls	787/1540	11/25	-			
		OR (95% CI)[e]	1.00	0.86 (0.42-1.76)	-	0.68	0.68	-
		Mean IGF-I [f]	242.8	255.3	-	0.28	0.28	-
		Mean IGFBP-3 [f]	3529	3748	-	0.06	0.06	0.46
	P0323 A/G	Cases/Controls	268/450	333/722	156/286			
		OR (95% CI)[e]	1.00	**0.77 (0.63-0.94)**	0.90 (0.70-1.16)	0.22	**0.03**	0.20
		Mean IGF-I [f]	241.8	246.5	245.4	0.30	0.16	0.83
		Mean IGFBP-3 [f]	3517	3544	3559	0.28	0.31	0.83
	P0322 G/T	Cases/Controls	333/657	352/682	96/186			
		OR (95% CI)[e]	1.00	1.02 (0.85-1.23)	1.01 (0.76-1.34)	0.88	0.84	1.00
		Mean IGF-I [f]	246.4	242.9	239.3	0.10	0.14	0.23
		Mean IGFBP-3 [f]	3548	3516	3541	0.56	0.35	
POU1F1	P0593 C/T	Cases/Controls	715/1416	61/118	3/2			
		OR (95% CI)e	1.00	1.04 (0.75-1.45)	3.01 (0.50-18.01)	0.53	0.66	0.23
		Mean IGF-I f	244.7	234.9	282.6	0.17	0.10	0.22
		Mean IGFBP-3 f	3541	3403	3132	0.004	0.006	0.20

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Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$ [b]	$P_{Dominant}$ [c]	$P_{Recessive}$ [d]
			Homozygous major	Heterozygous	Homozygous minor			
GHRH	P0342 A/G	Cases/Controls	745/1468	35/60	-			
		OR (95% CI)[e]	1.00	1.16 (0.75-1.79)	-	0.50	0.50	-
		Mean IGF-I [f]	243.4	248.8	-	0.45	0.45	-
		Mean IGFBP-3 [f]	3527	3533	-	0.93	0.93	-
GHRHR	P0347 C/T	Cases/Controls	719/1383	76/66	1/4			
		OR (95% CI)[e]	1.00	0.87 (0.65-1.16)	0.42 (0.05-3.77)	0.27	0.31	0.45
		Mean IGF-I [f]	242.6	244.7	268.1	0.49	0.57	0.38
		Mean IGFBP-3 [f]	3530	3541	3699	0.69	0.75	0.56
	P0348 G/A	Cases/Controls	719/1401	86/175	1/7			
		OR (95% CI)[e]	1.00	0.95 (0.72-1.24)	0.26 (0.03-2.09)	0.41	0.54	0.21
		Mean IGF-I [f]	243.2	245.1	251.8	0.60	0.63	0.72
		Mean IGFBP-3 [f]	3534	3529	3596	0.99	0.94	0.79
	P0353 T/C	Cases/Controls	434/824	305/612	47/111			
		OR (95% CI)[e]	1.00	0.94 (0.79-1.13)	0.81 (0.57-1.15)	0.24	0.36	0.29
		Mean IGF-I [f]	244.5	244.2	240.0	0.56	0.74	0.44
		Mean IGFBP-3 [f]	3532	3516	3648	0.33	0.91	**0.03**
	P0359 C/T	Cases/Controls	612/1226	157/292	10/13			
		OR (95% CI)[e]	1.00	1.08 (0.87-1.34)	1.56 (0.68-3.56)	0.29	0.38	0.31
		Mean IGF-I [f]	244.8	242.9	223.2	0.26	0.41	0.13
		Mean IGFBP-3 [f]	3528	3553	3396	0.84	0.64	0.32
	P0360 C/G	Cases/Controls	393/724	325/656	61/152			
		OR (95% CI)[e]	1.00	0.91 (0.76-1.09)	0.75 (0.54-1.03)	0.07	0.14	0.11
		Mean IGF-I [f]	244.5	244.2	241.3	0.61	0.76	0.54
		Mean IGFBP-3 [f]	3526	3523	3616	0.22	0.62	0.06

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Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$ [b]	$P_{Dominant}$ [c]	$P_{Recessive}$ [d]
			Homozygous major	Heterozygous	Homozygous minor			
SST	P0692 T/C	Cases/Controls	584/1196	181/282	11/24			
		OR (95% CI)[e]	1.00	**1.31 (1.06-1.61)**	0.95 (0.46-1.94)	**0.04**	**0.02**	0.72
		Mean IGF-I [f]	244.6	242.2	244.2	0.56	0.52	0.99
		Mean IGFBP-3 [f]	3520	3562	3526	0.31	0.26	0.98
	P0689 T/C	Cases/Controls	609/1236	158/252	9/14			
		OR (95% CI)[e]	1.00	**1.27 (1.02-1.59)**	**1.28 (0.55-2.97)**	**0.04**	**0.03**	0.66
		Mean IGF-I [f]	244.7	240.1	256.3	0.46	0.30	0.37
		Mean IGFBP-3 [f]	3521	3575	3621	0.12	0.12	0.52
SSTR1	P0694 T/C	Cases/Controls	348/661	317/632	81/140			
		OR (95% CI)[e]	1.00	0.95 (0.78-1.14)	1.10 (0.81-1.49)	0.87	0.78	0.42
		Mean IGF-I [f]	245.4	239.8	242.1	0.16	0.08	0.90
		Mean IGFBP-3 [f]	3546	3494	3544	0.35	0.13	0.64
SSTR2	P0837 A/G	Cases/Controls	314/661	339/599	83/158			
		OR (95% CI)[e]	1.00	1.20 (0.99-1.45)	1.12 (0.83-1.50)	0.16	0.08	0.87
		Mean IGF-I [f]	244.3	241.5	240.6	0.32	0.61	0.61
		Mean IGFBP-3 [f]	3527	3524	3489	0.53	0.43	0.43
	P0836 G/C	Cases/Controls	249/444	376/697	111/277			
		OR (95% CI)[e]	1.00	0.98 (0.80-1.19)	**0.74 (0.57-0.96)**	**0.04**	0.31	**0.02**
		Mean IGF-I [f]	239.5	244.4	244.4	0.18	0.61	0.61
		Mean IGFBP-3 [f]	3535	3542	3549	0.74	0.81	0.81
SSTR3	P0702 G/A	Cases/Controls	492/951	244/483	39/73			
		OR (95% CI)[e]	1.00	0.98 (0.81-1.18)	1.03 (0.69-1.54)	0.94	0.88	0.86
		Mean IGF-I [f]	243.5	243.4	242.2	0.88	0.92	0.84
		Mean IGFBP-3 [f]	3518	3537	3523	0.64	0.56	0.98

APPENDIX 4

Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$ [b]	$P_{Dominant}$ [c]	$P_{Recessive}$ [d]
			Homozygous major	Heterozygous	Homozygous minor			
SSTR4	P0710 T/G	Cases/Controls	280/549	377/715	112/243			
		OR (95% CI)[e]	1.00	1.04 (0.86-1.25)	0.89 (0.68-1.17)	0.59	0.98	0.28
		Mean IGF-I [f]	241.0	244.6	244.5	0.30	0.23	0.71
		Mean IGFBP-3 [f]	3527	3535	3523	0.99	0.86	0.84
SSTR5	P0719 C/A	Cases/Controls	631/1190	83/135	1/2			
		OR (95% CI)[e]	1.00	1.16 (0.87-1.55)	1.00 (0.09-11.03)	0.33	0.32	1.00
		Mean IGF-I [f]	243.3	240.0	204.4	0.34	0.40	0.27
		Mean IGFBP-3 [f]	3533	3532	3199	0.79	0.89	0.34
	P0723 C/T	Cases/Controls	654/1187	59/135	2/5			
		OR (95% CI)[e]	1.00	0.81 (0.59-1.11)	0.76 (0.13-4.40)	0.19	0.18	0.77
		Mean IGF-I [f]	242.1	249.5	250.8	0.13	0.13	0.76
		Mean IGFBP-3 [f]	3535	3508	3851	0.93	0.75	0.22
	P0727 T/C	Cases/Controls	210/394	339/643	166/290			
		OR (95% CI)[e]	1.00	1.00 (0.81-1.24)	1.09 (0.84-1.41)	0.56	0.80	0.48
		Mean IGF-I [f]	240.8	243.8	243.8	0.44	0.35	0.75
		Mean IGFBP-3 [f]	3555	3517	3534	0.54	0.30	0.94
	P0827 G/A	Cases/Controls	680/1255	33/69	2/3			
		OR (95% CI)[e]	1.00	0.86 (0.56-1.33)	1.14 (0.19-6.93)	0.58	0.53	0.87
		Mean IGF-I [f]	242.0	258.3	274.4	**0.01**	**0.01**	0.31
		Mean IGFBP-3 [f]	3533	3514	3666	0.93	0.85	0.67

[a] OR = Odds ratio; CI = confidence interval; IGF-I and IGFBP3 levels expressed in ng/mL; [b] P-value for codominant model (trend); [c] P-value for dominant model
[d] P-value for recessive model; [e] OR ; [f] Means adjusted for age and center

APPENDIX 4

Table 3a. Associations between haplotypes in *GH1* and breast cancer risk and IGF-I and IGFBP-3 levels[a] adjusted for age and center.

GH1 Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Reces
hACGG	0.33	OR (95% CI)[b]	1.00	1.00	1.0
		Alpha IGF-I (*P*) [c]	430.1	429.8	424
		Alpha IGFBP-3 (*P*) [c]	2563	2563	251
hTCAT	0.29	OR (95% CI)[b]	1.03 (0.88-1.21)	1.05 (0.86-1.28)	1.00 (0.7
		Beta IGF-I (*P*) [c]	**-5.43 (0.04)**	**-6.82 (0.03)**	-1.05 (
		Beta IGFBP-3 (*P*) [c]	-40.42 (0.11)	-59.77 (0.06)	31.74 (
hTCAG	0.17	OR (95% CI)[b]	1.13 (0.94-1.36)	1.13 (0.92-1.40)	1.29 (0.8
		Beta IGF-I (*P*) [c]	-0.43 (0.89)	1.28 (0.71)	-1.63 (
		Beta IGFBP-3 (*P*) [c]	-40.71 (0.18)	-25.30 (0.46)	-129.19
hTCGG	0.09	OR (95% CI)[b]	0.94 (0.73-1.22)	0.96 (0.73-1.26)	0.35 (0.0
		Beta IGF-I (*P*) [c]	-8.16 (0.05)	-5.95 (0.18)	**-46.80** (
		Beta IGFBP-3 (*P*) [c]	-78.16 (0.06)	-68.02 (0.12)	-289.47
hACAG	0.06	OR (95% CI)[b]	0.97 (0.71-1.34)	0.98 (0.70-1.38)	0.76 (0.2
		Beta IGF-I (*P*) [c]	-9.82 (0.06)	-9.47 (0.09)	-17.66 (
		Beta IGFBP-3 (*P*) [c]	-63.65 (0.22)	-62.74 (0.26)	-115.26
hACAT	0.03	OR (95% CI)[b]	1.01 (0.66-1.54)	1.03 (0.67-1.60)	-
		Beta IGF-I (*P*) [c]	-3.39 (0.63)	-1.48 (0.84)	3.02 ((
		Beta IGFBP-3 (*P*) [c]	-17.30 (0.80)	-1.20 (0.99)	81.32 (
hTCGT	0.02	OR (95% CI)[b]	1.02 (0.56-1.86)	1.01 (0.56-1.84)	-
		Beta IGF-I (*P*) [c]	-3.06 (0.76)	-1.91 (0.85)	-
		Beta IGFBP-3 (*P*) [c]	77.75 (0.43)	91.06 (0.36)	-

[a] OR = Odds ratio; CI = confidence interval
[b] Reference group = hACGG
[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of the SNPs defining the haplotypes is: P0320, P0327, P0323, P0322.

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Table 3b. Associations between haplotypes in *GHRHR* and breast cancer risk and IGF-I and IGFBP-3 levels[a] adjusted for age and center.

GHRHR Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Reces
hCGTCC	0.57	OR (95% CI)[b]	1.00	1.00	1.0
		Alpha IGF-I (*P*) [c]	426.9	426.3	425
		Alpha IGFBP-3 (*P*) [c]	2501	2508	251
hCGCCG	0.24	OR (95% CI)[b]	0.94 (0.81-1.10)	0.98 (0.81-1.19)	0.73 (0.4
		Beta IGF-I (*P*) [c]	-1.13 (0.65)	-0.47 (0.87)	-2.84 (
		Beta IGFBP-3 (*P*) [c]	32.59 (0.18)	21.51 (0.47)	121.82
hCGTTC	0.10	OR (95% CI)[b]	1.19 (0.95-1.49)	1.23 (0.95-1.59)	1.51 (0.6
		Beta IGF-I (*P*) [c]	-3.56 (0.30)	-2.69 (0.47)	-17.65 (
		Beta IGFBP-3 (*P*) [c]	21.85 (0.52)	26.46 (0.47)	-98.56 (
hTATCG	0.05	OR (95% CI)[b]	0.78 (0.59-1.04)	0.79 (0.59-1.07)	0.67 (0.0
		Beta IGF-I (*P*) [c]	2.03 (0.65)	1.55 (0.74)	14.86 (
		Beta IGFBP-3 (*P*) [c]	29.71 (0.51)	22.87 (0.62)	184.58
hCGCCC	0.02	OR (95% CI)[b]	1.14 (0.72-1.82)	1.15 (0.72-1.84)	-
		Beta IGF-I (*P*) [c]	-7.07 (0.35)	-6.77 (0.37)	-
		Beta IGFBP-3 (*P*) [c]	-3.02 (0.97)	-6.34 (0.93)	-

[a] OR = Odds ratio; CI = confidence interval
[b] Reference group = hACGG
[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of the SNPs defining the haplotypes is: P0347, P0348, P0353, P0359, P0360.

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Table 3c. Associations between haplotypes in *SST* and breast cancer risk and IGF-I and IGFBP-3 levels[a] adjusted for age and center.

SST Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Reces
hTT	0.88	OR (95% CI)[b]	1.00	1.00	1.0
		Alpha IGF-I (*P*) [c]	425.71	425.87	425.
		Alpha IGFBP-3 (*P*) [c]	2522	2521	252
hCC	0.10	OR (95% CI)[b]	**1.24 (1.01-1.52)**	**1.27 (1.02-1.59)**	1.21 (0.5
		Beta IGF-I (*P*) [c]	-2.26 (0.50)	-3.50 (0.34)	12.56 (
		Beta IGFBP-3 (*P*) [c]	53.99 (0.11)	57.71 (0.11)	89.47 (
hCT	0.02	OR (95% CI)[b]	1.06 (0.66-1.70)	1.08 (0.67-1.75)	-
		Beta IGF-I (*P*) [c]	0.30 (0.97)	0.57 (0.94)	-21.02 (
		Beta IGFBP-3 (*P*) [c]	-92.08 (0.23)	-94.48 (0.23)	-119.71

[a] OR = Odds ratio; CI = confidence interval
[b] Reference group = hACGG
[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of the SNPs defining the haplotypes is: P0692, P0689.

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Table 3d. Associations between haplotypes in *SSTR2* and breast cancer risk and IGF-I and IGFBP-3 levels[a] adjusted for age and center.

SSTR2 Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Reces
hAC	0.43	OR (95% CI)[b]	1.00	1.00	1.0
		Alpha IGF-I (*P*) [c]	426.01	424.59	423.
		Alpha IGFBP-3 (*P*) [c]	2526	2513	252
hGG	0.33	OR (95% CI)[b]	1.15 (0.99-1.33)	**1.24 (1.03-1.51)**	1.03 (0.7
		Beta IGF-I (*P*) [c]	-2.58 (0.28)	-2.12 (0.49)	-3.36 (
		Beta IGFBP-3 (*P*) [c]	-10.67 (0.65)	-3.01 (0.92)	-33.05 (
hAG	0.24	OR (95% CI)[b]	1.13 (0.96-1.33)	1.19 (0.99-1.45)	0.96 (0.6
		Beta IGF-I (*P*) [c]	-2.19 (0.40)	-0.03 (0.99)	-9.98 (
		Beta IGFBP-3 (*P*) [c]	3.80 (0.88)	21.25 (0.49)	-43.85 (

[a] OR = Odds ratio; CI = confidence interval
[b] Reference group = hACGG
[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of the SNPs defining the haplotypes is: P0837, P0836.

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Table 3e. Associations between haplotypes in *SSTR5* and breast cancer risk and IGF-I and IGFBP-3 levels[a] adjusted for age and center.

SSTR5 Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Reces
hCCTG	0.54	OR (95% CI)[b]	1.00	1.00	1.0
		Alpha IGF-I (*P*) [c]	426.9	427.1	429
		Alpha IGFBP-3 (*P*) [c]	2515	2531	25(
hCCCG	0.33	OR (95% CI)[b]	1.05 (0.91-1.22)	1.05 (0.86-1.27)	1.12 (0.8
		Beta IGF-I (*P*) [c]	-0.28 (0.90)	-0.51 (0.87)	-1.35 (
		Beta IGFBP-3 (*P*) [c]	-14.98 (0.51)	-35.17 (0.24)	22.41 (
hACCG	0.06	OR (95% CI)[b]	1.29 (0.85-1.96)	1.32 (0.85-2.05)	1.00 (0.9
		Beta IGF-I (*P*) [c]	-2.23 (0.63)	-1.69 (0.72)	-38.72 (
		Beta IGFBP-3 (*P*) [c]	-9.74 (0.84)	-7.86 (0.87)	-329.41
hCTCG	0.05	OR (95% CI)[b]	0.82 (0.60-1.13)	0.81 (0.58-1.11)	0.78 (0.1
		Beta IGF-I (*P*) [c]	7.25 (0.13)	7.69 (0.13)	7.87 ((
		Beta IGFBP-3 (*P*) [c]	-11.07 (0.82)	-25.04 (0.62)	321.08
hCCCA	0.03	OR (95% CI)[b]	0.90 (0.60-1.34)	0.87 (0.57-1.33)	1.21 (0.2
		Beta IGF-I (*P*) [c]	**16.32 (0.01)**	**17.05 (0.01)**	31.37 (
		Beta IGFBP-3 (*P*) [c]	-12.49 (0.84)	-21.66 (0.75)	138.06

[a] OR = Odds ratio; CI = confidence interval
[b] Reference group = hACGG
[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of the SNPs defining the haplotypes is: P0719, P0723, P0727, P0827.

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Supplementary Table 1. PCR primers, probes and labels.

Gene	Polymorphism[a]	Probes	PCR Primers
GHRH	***GHRH1***		
P0342 A/G	rs4988491	VIC-MGB-ATGTTAGCTTTAAAAAA FAM-MGB-TGTTAGCTTCAAAAAA	AGCTGCCTGGTTTGCACTCT TCCCAAAGTGGTATTACGAGGTGTA
GHRHR	***GHRHR***		
P0347 C/T	rs4988495	HEX-LNA-TCCTCCAGCTTCACCC FAM-LNA-CCTCCAGCCTCACCC	TAGGAGGCAGGTGGTGGCTT CCAGGAGCAGCAGAGACTCAG
P0348 G/A	rs4988496	HEX-LNA-AGGCTGCCCTGCGACCTGGGA FAM-LNA-AGGCTGCCCTACGACCTGGGA	CTCTGCTGCTCCTGGCTCTC GCACCCCTCACCTGACTCTG
P0353 T/C	rs4988501	VIC-MGB-AGTTTGATTCGATTCAC FAM-MGB-ATTCGGTTCACCTCC	TCAAGTTCAGCTCAATTCAATTCAG GGATTCTGGTGTGCTGCAACT
P0359 C/T	rs4988503	VIC-MGB-CTTGGGAGCCTAGGA FAM-MGB-AGTCTTGGAAGCCTAG	AAGTGCACACGACAGTTTCTAATCC CGCCTGCAGGAAAGACAAA
P0360 C/G	rs4988505	VIC-MGB-TGAACCGGAATGTT FAM-MGB-TGAACCCGAATGTT	CTGTCCTGAGCTTCTGGATCAAG TCAGAGGAAGGATGGATAAGAGATG
GH1	***GH1***		
P0322 G/T	rs2005172	HEX-TCCCACTGTTGACCCCACCTGTTT FAM-TCCCACTGTTGCCCCCACCTGTTT	CATTAGCACAAGCCCGTCAGT CCTTTTTATACCCTGGCCCCTTC
P0323 A/G	rs6171	HEX-LNA-AC+AA+GAG+ACC+AG+CTC FAM-LNA-AC+AA+GAG+ACC+GG+CTC	AGAGAAGGGGCCAGGGTATAAA CTAGGTGAGCGTCCACAGGA
P0327 C/A	rs6173	HEX-LNA-CCATTGCCGCTAGGTGA FAM-LNA-CCATTGCAGCTAGGTGA	GACACATTGTGCCCAAAGG CAGCTCCAGCATCCCAAG
P0320 T/A	rs2665802	HEX-LNA-TAG+CAG+ACC+AGG+CCCTG FAM-LNA-TAG+CAG+TCC+AGG+CCCTG	ACTTTGAGAGCTGTGTTAGAGAAAC TTCACGAGGGGAAATGAAGAATAAG
GHR	***GHR***		
P0329 G/A	rs6179	VIC-MGB-CATCCATCCTTTCTG FAM-MGB-ATCCACCCTTTCTG	ATATCCAAGTGAGATGGGAAGCA CCATTTAGTTTCATTTACTTCTTTGTA TTGA
P0332 G/T	rs6182	HEX-CATGATGCTTTCCCTGCTACTCAGC FAM-CATGATGCTTGCCCTGCTACTCAGC	TTATGCCTTGACCAGAAGAATCAAA GTTTTTCTCTGCTTGGATAACACTG
P0335 T/G	rs6180	VIC-MGB-AGTGCCTCCCTGTGG FAM-MGB-ATCCACCCTTTCTG	TTATGGACAATGCCTACTTCTGTGA GCTGTATGTGTGATTCAACCTTGAT
SST	***SST***		
P0692 T/C	rs4988513	HEX-LNA-AGCCCTCCCTAAGCCTTG FAM-LNA-CCCTCCCTGAGCCTTGC	TCTTTAGAAGGACTGAGCATCCCT CAGGTAAGGAGACTCCCTCGAC
P0689 T/C	P0689[c]	VIC-MGB-CTGGAGAATCCGGG FAM-MGB-CTGGAGGATCCGGG	GGTAAGGAGACTCCCTCGACGT GGCAGGAGCAAGGCTTAGG
SSTR1	***SSTR1***		
P0694 T/C	rs2228497	HEX-AGCTGGTCAACGTGTTTGCTG FAM-AGCTGGTTAACGTGTTTGC	CGCAAGATCACCTTAATGG CGAGGATGACCGACAG

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SSTR2	***SSTR2***		
P0837 A/G	rs998571	VIC-MGB-CTGAGAGGCTAAACCAGAAA FAM-MGB-AGAGGCTAAGCCAGAAA	AACTCTAGAGCTTAATGTTGATGTGG AAA CGTCGGTCACAGGCTGTTTAA
P0836 G/C	rs1466113	HEX-LNA-TGGTGAGACTTTAAACA FAM-LNA-TGGTGAGAGTTTAAACA	AGAGCTTAATGTTGATGTGG CCTTAATGGACCCTGGAG
SSTR3	***SSTR3***		
P0702 G/A	rs229569	VIC-MGB-CGAAGAGCGCCACC FAM-MGB-CGAAGAGTGCCACCAC	GGGTCACGCGCATGGT CACCACGTTGACGATGTTGAG
SSTR4	***SSTR4***		
P0710 T/G	rs3746726	VIC-MGB-CACGAAGAGGTTCAG FAM-MGB-CACGACGAGGTTC	TCTGCTGGATGCCTTTCTACGT TGGTTGACGGTGGCATCA
SSTR5	***SSTR5***		
P0719 C/A	rs4988483	VIC-MGB-CAGCAGGTACAGCAC FAM-MGB-CAGCATGTACAGCAC	CTGGAGGCGGTGACAACAG CCCGGCCGCACACA
P0723 C/T	rs4988487	HEX-LNA-CCCTTCGGCTCCGT FAM-LNA-CCTTCGGCCCCGT	CTACATTCTCAACCTGGC GCAGAAGACACTGGTGAAC
P0727 T/C	rs169068	HEX-CGCCACGGAGCTGCGTCCAGACA FAM-CGCCACGGAGCCGCGTCCAGACA	AAGGGCTCTGGTGCCAAGGA CACTCTCACAGCTTGCTGGTCT
P0827 G/A	rs642249	HEX-AGGCCACGCCGCCCGCGCAC FAM-AGGCCACGCCACCCGCGCAC	CTGCGTCCAGACAGGATCCG CACTCTCACAGCTTGCTGGTCT
POU1F1	***POU1F1***		
P0593 C/T	rs300982	HEX-LNA-AC+TAGCG+TGCA+CC FAM-LNA-TACT+AGCG+CGC+ACCC	AAAGGGATTTCCTTGCAGTA AGCTTGGCAACTCATTCC

[column in red will be omitted from the final version of the manuscript. It is left temporarily for ease of consultation]

[a] Polymorphisms are identified by their dbSNP accession number. dbSNP is accessible at http://www.ncbi.nlm.nih.gov/SNP/

[b] Fluorescent dye and presence of stabilizing molecules (MGB or LNA) are indicated. For LNA probes, a plus sign indicates bases modified with an LNA molecule

[c] P0689 is an internal reference. This polymorphism is not represented in dbSNP. See ref. Naylor *et al.*, 1983

APPENDIX 5

Polymorphisms of genes coding for insulin-like growth factor 1 and its major binding proteins, circulating levels of IGF-I and IGFBP-3 and breast cancer risk: results from the EPIC study

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Abstract

Insulin-like growth factor I (IGF-I) stimulates cell proliferation and can enhance the development of tumors in different organs. Epidemiological studies have shown that an elevated level of circulating IGF-I is associated to increased risk of breast cancer, as well as of other cancers. Most of circulating IGF-I is bound to an acid labile subunit and to one of six insulin-like growth factor binding proteins (IGFBPs), among which the most important are IGFBP-3 and IGFBP-1. Genetic variants of the *IGF1* gene and of genes encoding for the major IGF-I carriers may predict circulating levels of IGF-I and have an impact on cancer risk. We tested this hypothesis with a large case-control study of 807 breast cancer patients and 1588 matched control subjects, nested within the European Prospective Investigation into Cancer and Nutrition. We genotyped 23 common single nucleotide polymorphisms in *IGF1*, *IGFBP1*, *IGFBP3* and *IGFALS*, and, in parallel, we measured serum levels of IGF-I and IGFBP-3 in samples of cases and controls. The main findings of our study were a weak but nominally significant association of a block of polymorphisms located at the 5' of the *IGF1* gene with breast cancer risk, particularly among women younger than 55, and a strong association of polymorphisms located in the 5' region of *IGFBP3* with circulating levels of IGFBP-3, which confirms previous findings. Our conclusion is that common genetic variation in the four candidate genes analyzed does not play a major role in altering breast cancer risk.

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Introduction

Insulin-like growth factor-I (IGF-I) is a peptide hormone that is derived principally from the liver and exerts mitogenic and metabolic activities that are regulators of growth, survival and cell differentiation in a number of cell and tissue types. An association between circulating IGF-I levels and breast cancer has been found in a number of epidemiologic studies. There has been consistent evidence of a role for high levels of IGF-I in breast cancer development, particularly among premenopausal women (Peyrat *et al.*, 1993; Bruning *et al.*, 1995; Bohlke *et al.*, 1998; Hankinson *et al.*, 1998; Toniolo *et al.*, 2000, Muti *et al.*, 2002; Yu *et al.*, 2002). High circulating IGF-I has been suggested to be a cancer risk factor also for other organs, such as the prostate (Mantzoros *et al.*, 1997; Chan *et al.*, 1998; Stattin *et al.*, 2000; Wolk *et al.*, 1998) and the colorectum (Ma *et al.*, 1999; Giovannucci *et al.*, 2000; Kaaks *et al.*, 2000; Palmqvist *et al.*, 2002). The role of IGF-I in cancer has been recently reviewed (Pollak *et al.*, 2004).

Most of circulating IGF-I is bound by one of six high affinity binding proteins which regulate IGF-I activity. About 90% of IGF-I is bound to insulin-like binding binding protein-3 (IGFBP-3) which forms a ternary complex with acid labile subunit (ALS). Most of the remaining IGF-I not bound to IGFBP-3 is bound one of the other five binding proteins (IGFBP1-2, IGFBP4-6) found in circulation. The smaller complexes are able to pass the vascular endothelial barrier and therefore may be important modulators of IGF-I activity at the cellular level (Rajaram *et al.*, 1997; Jones JI, 1993 Proc Natl Acad Sci USA;90:10553-7). There is physiologic evidence of a role for IGFBPs to work either in an IGF-I-dependent, such as increasing the half-life of IGF-I and modulating access to the IGF-I receptor, or IGF-I-independent fashion by mediating their effects directly on target cells, where they generally have a proapoptotic role (Mohan S, 2002 J Endocrinol;175:19-31; Jones JI, 1993 Proc Natl Acad Sci USA;90:10553-7; Perks *et al.*, 1999, Gleeson *et al.*, 2001).

While nutrition is a key determinant of circulating IGF-I levels (Thissen *et al.*, 1994; Kaaks & Lukanova 2002), heritability studies have shown that a up to 40-60% of the variation in circulating IGF-I levels is determined by genetic factors (Hall *et al.*, 1999; Hong *et al.*, 1997; Harrela *et al*, 1996). Therefore, there is merit in searching for the genetic factors that influence circulating IGF-I levels and research to identify genetic determinants of circulating IGF-I is intensifying (Lopez-Bermejo *et al.*, 2000; Jernstrom *et al.*, 2001; Hasegawa *et al.*, 2000; Vaessen *et al.*, 2001).

For the present study, we focused on 23 common single nucleotide polymorphisms (SNPs) identified from the public data bases that have the highest chance of having an impact on gene expression or function of *IGF1* and those genes involved in its transport, *IGFBP1*, *IGFBP3* and *IGFALS*. Genetic variation affecting the function of these genes may influence IGF-I function

directly or indirectly via circulating levels of IGF-I and its major carrier IGFBP-3. To assess this we conducted a large case-control study of 807 breast cancer patients and 1588 matched control subjects, nested within the cohorts of the European Prospective Investigation into Cancer and Nutrition (EPIC) (Riboli *et al.*, 2002; Bingham and Riboli, 2004) and correlating presence of these polymorphisms with circulating IGF-I and IGFBP-3 levels and breast cancer risk.

Material and methods

The EPIC study

The EPIC cohort consists of about 370,000 women and 150,000 men, aged 35-69, recruited between 1992 and 1998 in 23 research centres in 10 Western European countries (Riboli *et al.*, 2002; Bingham & Riboli, 2004). The vast majority (>97%) of subjects recruited in the EPIC cohort are of European ("Caucasian") origin. All EPIC study subjects provided anthropometric measurements (height, weight, waist & hip circumferences), and extensive, standardized questionnaire information about medical history, diet, physical activity, smoking, and other lifestyle factors. Women also answered questions about menstrual and reproductive history, hysterectomy, ovariectomy, and use of exogenous hormones for contraception or treatment of menopausal symptoms. In addition, about 240,000 women and 140,000 men provided a blood sample, and plasma, serum, red cells, and a buffy coat were stored for future analyses on cancer cases and controls. Cohort members are contacted regularly to renew information on smoking, alcohol drinking, physical activity, weight, menstrual history, pregnancies, use of medications and exogenous hormones, hysterectomy, and first development of major diseases other than cancer (e.g. diabetes).

Cases of cancer occurring after recruitment into the cohort are identified through local and national cancer registries in 7 of the 10 countries, and in France, Germany, and Greece by a combination of contacts with national health insurances and/or active follow-up through the study subjects or their next of kin. Follow-up on vital status, to monitor the population remaining at risk for cancer, is achieved through record linkage with mortality registries. In all EPIC study centers, cancer diagnosis is confirmed through comprehensive review of pathology reports, and checks for completeness of follow-up are made regularly. A fully detailed description of the EPIC study has been published elsewhere (Riboli *et al.*, 2002; Bingham and Riboli, 2004).

Selection of case and control subjects.

Cases and controls from the present study were from 16 of the 23 EPIC recruitment centers, in 7 of the 10 countries (U.K., Germany, Netherlands, France, Spain, Italy and Greece), and most were also

part of nested case-control studies on serum hormones and breast cancer risk reported in detail elsewhere (Kaaks *et al.*, 2 manuscripts submitted, Rinaldi *et al.*, manuscript in preparation).

Case subjects were selected among women who developed breast cancer after their recruitment into the EPIC study, and before the end of the study period, for each study center defined by the latest end-date of follow-up. Women who used any hormone replacement therapy at the time of blood donation, or any exogenous hormones for contraception or medical purposes, and who had previous diagnosis of cancer (except non-melanoma skin cancer) were excluded from the study, because each of these various factors could have altered circulating hormone levels.

For each case subject with breast cancer, two control subjects were chosen at random from among cohort members alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. Control subjects were matched to the cases by study center where the subjects were enrolled in the cohort, as well as by menopausal status (pre-menopausal, post-menopausal, peri-menopausal/undefined), age (± 6 months) at enrollment, follow-up time (Kaaks *et al.*, manuscripts submitted), fasting status, time of the day of blood donation, phase of the menstrual cycle for premenopausal women.

Approval for the study was given by the relevant Ethical Committees, both at the IARC and in the EPIC recruitment centers.

Identification and selection of SNPs

We collected data on polymorphisms from publicly available databases, such as dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), SNPper (http://snpper.chip.org/) and Frequency Finder (http://bluegenes.bsd.uchicago.edu/frequencyfinder/). We complemented database searches with literature review and, for *IGFBP1* and *IGFBP3*, with analysis of 95 subjects from the EPIC population by denaturing high-performance liquid chromatography (DHPLC; Xiao and Oefner, 2001).

To be included in the study, polymorphisms had to be located in exons (including untranslated regions), exon-intron junctions, or promoter regions of a gene of interest, or otherwise should be within intronic regions that showed greater than 80% homology between human and mouse (as reported by the UCSC Genome Browser, http://genome.ucsc.edu/), and thus were likely to harbor regulatory sequences. In addition, we also included polymorphisms with documented evidence of their existence in Caucasians, either according to literature data or to our own experimental analysis by DHPLC. Among all polymorphisms thus identified, we only retained those with a minor allele frequency ≥5% in Caucasians, or those that result in an aminoacid change and had a minor allele frequency ≥1%. Finally, we particularly favored the inclusion of all

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polymorphisms previously reported in the literature to be associated with cancer and/or levels of circulating hormones.

Genotyping

Buffy coat samples for the study subjects were retrieved from the EPIC biorepository and DNAs were extracted on an Autopure instrument (Gentra Systems, Minneapolis, MN) with Puregene chemistry (Gentra Systems, Minneapolis, MN).

Genotyping was performed by the 5' nuclease assay (TaqMan). The order of DNAs from cases and controls was randomized on PCR plates in order to assure that an equal number of cases and controls could be analyzed simultaneously. Taqman probes were synthesized by either Applied Biosystems (with MGB chemistry) or Proligo (with or without LNA chemistry). Sequences of primers and probes are reported in Supplementary Table 1. The reaction mix included 10 ng genomic DNA, 5 pmol of each primer, 1 pmol of each probe, and 2.5 µl of 2 X master mix (Applied Biosystems) in a final volume of 5 µl. The thermocycling included 50 cycles with 30 seconds at 95 °C followed by 60 seconds at 60 °C. PCR plates were read on an ABI PRISM 7900HT instrument (Applied Biosystems). In order to validate genotype identification we repeated 8% of all genotypes. Laboratory personnel was kept blinded to case-control status throughout the study.

Hormone measurements

Measurements of IGF-I and IGFBP-3 were performed in the laboratory of the Hormones and Cancer, at IARC, using enzyme-linked immunosorbent assays (ELISA) from Diagnostic System Laboratories (DSL, Webster, Texas). The IGF-I assays included an acid-ethanol precipitation step to eliminate IGF-I binding proteins, to avoid their interference with the IGF-I measurement. Measurements were performed on never thawed serum sample aliquots. The mean intra-batch and inter-batch coefficients of variation were 6.2% and 16.2% respectively for IGF-I, and 7.2% and 9.7% respectively for IGFBP-3.

Statistical analysis

Invidual haplotype frequencies (i.e., estimated numbers of copies of haplotypes) were reconstructed using the program "tagSNPs" (http://www-rcf.usc.edu/~stram/tagSNPs.html) by Stram *et al.* (Stram *et al.*, 2003; Stram *et al.*, 2003). This program calculates, for each individual, the expected numbers of copies ("dosages") of each of the haplotypes compatible with the individuals' SNP genotypes. This method takes account of uncertainties in the haplotype reconstruction for individuals that are

heterozygote for 2 or more of the SNPs within a given gene. Haplotype dosages are estimated from the individuals' SNP genotype data, and from overall haplotype frequency estimates for the full study population (cases and controls combined) estimated by a maximum likelihood method. For each haplotype, the dosage values range from 0 to 2.0 (alleles), and for each individual these dosage values add up to a total value of 2.0 across all possible haplotypes.

All association analyses, at the level of individual SNPs or gene loci, were performed under different assumed modes of inheritance of effect – dominant, recessive, or co-dominant – associated with alleles. In the 'dominant' model, circulating peptide levels or disease risks were compared between subjects carrying at least one copy of the rare allele, and those who had none; in the 'recessive' model, the comparison was between those who were homozygous for the rare allele, and all others; in the 'codominant' model, individuals' peptide levels or the logarithm of disease risk were linearly related to the number of copies of an allele (0, 1, or 2 for SNP alleles, or dosages for the haplotype) carried by the individuals. For rare alleles, with a frequency less than 20% (i.e., a prevalence of homozygous recessive allele carriers less than 4.0%), only the dominant model was used. To test whether any association of gene variants with breast cancer risk could be mediated by alterations in circulating levels of IGF-I and/or IGFBP-3, these associations were also estimated with adjustment for serum peptide levels.

Relationships of polymorphic gene variants with serum levels of IGF-I and IGFBP-3 were estimated by standard normal regression models, stratified by EPIC recruitment center and further adjusted for age. Relationships of polymorphic variants with breast cancer risk (odds ratios (OR)) were estimated using conditional logistic regression models, applied on the matched case-control sets. Both series of analyses were performed at the level of single SNP loci, as well as at the level of haplotypes (using the haplotype dosage values). Haplotype analyses were performed at the level of full gene loci – i.e., including haplotypes based on all of the SNPs in that gene – and in a few cases also at the level of well-delineated haplotype blocks within a gene. In all haplotype analyses, the most common haplotype was used as the reference category.

The program Haploview (Barrett *et al.*, 2004; http://www.broad.mit.edu/personal/jcbarret/haploview/) was used to prepare visual diagrams of LD patterns and to examine possible LD block structures within each gene. Block boundaries were determined using the criterion of Gabriel *et al.* (Gabriel *et al.*, 2002), who utilized 90% confidence limits of D' to define sites of historical recombination between SNPs.

Subgroup analyses on women with a breast cancer diagnosis either before (45% of the subjects), or after age at diagosis of 55 years (the age at which over 99% of women enrolled in the EPIC cohort

declared themselves menopausal) were used to examine whether associations of gene variants with breast cancer risk differed between women with cancer an approximately premenopausal or postmenopausal age, and possible heterogeneity of effect between these two age groups was tested using a chi square test.

Results

Eight hundred seven incident cases of breast cancer from the EPIC cohort and 1588 matched controls were included in our study. The mean age of study subjects was 55 years (5^{th}-95^{th} percentile: 39.9-68.7 years). For cases, the mean age at diagnosis was 57 years (5^{th}-95^{th} percentile: 42-71 years). Based on the questionnaire data, 32% of the subjects were pre-menopausal at blood donation, 10% were peri-menopausal or of unknown menopausal status, and 58% were post-menopausal. Cases had a significantly lower number of full-term pregnancies than controls (means: 2.35 *vs.* 2.47, p=0.02) and were significantly older at first full-term pregnancy (26 years *vs.* 25.5 years in controls, p=0.02). Age at menarchy did not differ between cases and controls, nor did body mass index. Serum levels of IGF-I were not significantly different between cases and controls (means: 242 ng/ml *vs.* 237.5 ng/ml, p=0.3), whereas cases had more elevated levels of serum IGFBP-3 than controls (means: 3016 ng/ml *vs.* 2936 ng/ml, p=0.02). The difference in IGFBP-3 levels was not observed in the subgroup of women younger than 55, and it was entirely confined to the older age group. Details on the relationships of IGF-I and IGFBP-3 with breast cancer risk analyses will be reported elsewhere (Rinaldi *et al.*, in preparation).

We collected information on polymorphisms from the literature, public databases and our own experimental analyses by DHPLC. This provided a list of 30 SNPs. By applying the selection criteria outlined in the Methods section, we selected 26 SNPs for genotyping. For 1 SNP, a genotyping assay could not be designed and for 2 more SNPs, TaqMan assays were generated but provided poor genotyping results. This left 23 polymorphisms that were genotyped on the DNAs of cases and controls (Table 1). The number of SNPs typed per gene ranged from three to eight. Genotyping call rates ranged between 95.27% and 99.44%. The distibutions of genotypes of all polymorphisms were in agreement with Hardy-Weinberg equilibrium. Repeated quality control genotypes (8% of the total) showed greater than 99% concordance for all assays. Results of associations between individual SNPs and cancer risk and circulating hormone levels are reported in Table 2. Tables 3a to 3d report results of analyses of haplotypes.

In the *IGF1* gene, we noted an association of the P0416 SNP, with the G/G genotype being protective for risk of breast cancer (p=0.05 and also a significant test for tend (p=0.03), indicating a modest effect in the heterozygote. Interestingly, when the cases were stratified by age of onset (less 55 years old or greater than or equal to 55 years old), this protective effect appears to be confined to

early age of onset cases, with a strong protective effect being observed odds ratio of 0.17 (CI 0.07-0.56) in the homozygous G/G (p = 0.005), there was also a protective effect under dominant (p=0.01) and codominant (p=0.002) models. LD studies of this region shows that there is strong LD between the P0416 and the two surrounding SNP's, P0416 and P0419. As expected, P0419 and P0415 also showed a protective effect among the entire set, although the only statistically significant results was P0419 when testing for trend. However as with P0416, the protective effect was statistically significant in both P0419 and P0415 in the breast cancer cases with an age of onset less than 55. Haplotype analysis of the *IGF1* data did not show clear associations. However, based on our control genotypes we noted a clear haplotype block that contains these three SNPs (Figure 1), therefore we restricted our haplotype analysis to only this block which (??). We also noted a significant decrease in circulating IGFBP-3 levels with the rare homozygous states for both P0416 and P0419 (again representing the LD between these markers). In contrast to risk with the same markers, this effect appeared restricted to a later age of onset in our age-stratified analysis. The only other result of interest in the *IGF1* gene was a relatively modest dominant effect in the polymorphsism P0425 and a higher mean circulating IGF-I level.

The polymorphism in the promoter of *IGFBP3* at position –202 has been associated with an increased mean circulating level of IGFBP-3. We show clearly the same effect between carrying the A allele of this polymorphism (P0832) and increased mean levels of circulating IGFBP-3 levels, providing further evidence for this association (p=<0.0001). Reflecting the strong amount of linkage disequilibrium (LD) in this area (Figure 2), we also noted a strong association between increased IGFBP-3 levels and alleles of the surrounding polymorphisms, P0845, P0844, P0450. Interestingly, this association was less obvious when observing on a gene-wide haplotype scale (table 3), but haplotype block calculations using the control genotypes show that as with *IGF1* a clear haplotype block exists across the promoter of *IGFBP3*. This analysis using only polymorphisms inside the haplotype block showed the association clearly.

The polymorphism P0453 of *IGFBP3* also showed a slight increase in the mean circulating IGF-I protein levels and one *IGFBP3* haplotype showed an increase in risk of breast cancer, under a recessive model.

IGFBP1 did not show associations with any of the endpoints. Polymorphism P0828 of *IGFALS* showed a significant correlation between the homozygous rare allele and a decrease in circulating mean IGF-I levels.

Discussion

The IGF-I pathway is involved in several growth and regulatory pathways and can act as a potent mitogen. The ability of IGF-I to affect breast cancer risk is not only dependent on the circulating

levels of IGF-I, but may also be affected by local expression of the high-affinity binding proteins. Among them, IGFBP-1 and IGFBP-3 are the most interesting. Both have been reported to be present at lower levels in the circulation in breast cancer cases when compared to controls (Kaulsay *et al.*, 1999). Higher IGFBP-1 levels have also been reported to be associated with reduced risk of breast cancer recurrence and death (Goodwin PJ, 2003 Breast Cancer Res Treat;74:65-76). IGFALS has never been studied in relation to cancer risk, but it is the major carrier of IGF-I in the bloodstream, together with one of the IGFBPs.

We have performed a large-scale association study, nested in the EPIC cohort, to assess the role of genetic variation of *IGF1* and of the genes encoding the major IGF-I binding proteins on risk of breast cancer and on circulating levels of IGF-I and IGFBP-3. The main advantages of our study are the large sample size (807 case and 1588 controls) and its prospective nature, which allowed us to measure prediagnostic hormone levels.

We genotyped 23 SNPs in the four candidate genes. Our criteria for selecting the SNPs to study were proven existence in the Caucasian population, high allele frequency and/or high chance of having an impact on gene expression or function of the gene product. We have not used a haplotype tagging approach, because we estimated that publicly available information is insufficient to select haplotype tagging SNPs. For instance, as of the time of writing this report, only five polymorphisms have been typed in the context of the HapMap project (the largest public initiative aimed at establishing genome-wide haplotype maps) in the region of *IGFBP1*. Three of them (rs1065780, rs4988515 and rs4619) coincide with SNPs we have studied. As for the others, one (rs3763497) is located in the immediate vicinity of a SNP we have typed (rs 1995051) and one (rs1065782) has been reported to have a very low frequency (minor allele frequency <0.01) in CEPH subjects. Clearly, the present coverage of this gene by the HapMap project is insufficient to do a proper selection of haplotype tagging SNPs, and based on the data currently available, we would have selected a set of SNPs not significantly different from the one we used. Likewise, only 2 SNPs have been typed by HapMap in *IGFBP3*, and 2 in *IGFALS*. Thirty-three SNPs have been genotyped by HapMap in the region of *IGF1*, which spans 85 kb. However, 17 of them were reported to have small allele frequencies, and many are located in the middle of large introns, which have no evidence of evolutionary conservation.

A complete resequencing of the gene region in order to establish a complete catalogue of polymorphisms and to reconstruct haplotypes goes beyond the scopes of the present project. On the other hand, it is unlikely that new common polymorphisms, which were the focus of our investigation, would be discovered by sequencing.

We have not typed a CA repeat polymorphism located in the 5' region of *IGF1*, which has been frequently included in previous studies. Several epidemiological studies on the association

between this polymorphism and breast cancer risk or IGF-I levels (Yu *et al.*, 2001; Jernstrom *et al.*, 2001a; Missmer *et al.*, 2002; Figer *et al.*, 2002; DeLellis *et al.*, 2003) have yielded highly conflicting results. This microsatellite has a large number of alleles, which results in fractioning the study population into a large number of classes. Even with a large sample size, this makes statistical analysis very difficult. Moreover, there are no studies that show a functional role for this microsatellite. We think that the *IGF1* CA repeat does not have a functional role of its own, and that associations reported with this microsatellite are likely to reflect LD with the SNPs we have included in our study.

The use of a multicentric study raises the possibility of confounding by population stratification. Over 97% of EPIC subjects are estimated to be of Caucasian origin. Nevertheless, there could still be confounding if the region of origin is associated both with the endpoint(s) under investigation (breast cancer risk and hormone levels) and with the exposure of interest (SNPs in our case). While different countries representede in EPIC do not have the same breast cancer incidences, this was not paralleled by wide differences of allele frequencies between subjects of different countries in our study (data not shown). In addition, we used recruitment center as a variable to match the controls to the breast cancer cases, and to adjust all analyses of association between SNPs and hormone levels. We believe therefore that population stratification should not be an issue in our study.

We have found an association with decreased breast cancer risk of a haplotype of *IGF1*. The haplotype is based on three SNPs in strong LD (Figure 1), located in the promoter and intron 1 of the gene. This association was particularly strong among women younger than 55.

No other variant among the ones we studied showed any association with cancer risk, except for two haplotypes of IGFBP3, one associated with increased risk and another with reduced risk. These associations are likely to be chance findings, as they are based on small numbers, and nothing was observed at the SNP level.

SNPs we have studied do not explain a large fraction of variability in IGF-I levels. There was a modest but significant association with increased levels of the same *IGF1* haplotype that was associated with cancer risk. It is difficult to interpret these associations, as these SNPs do not have an obvious functional role. To the best of our knowledge, nothing is known about what genomic elements are involved in the regulation of IGF1 transcription, except for a region immediately upstream of the transcription start (Porcu *et al.*, 1994; Rubini *et al.*, 1994), which however harbors no known genetic variation. It is thus possible that the SNPs we have studied in the 5' region of the gene are influencing its regulation, but functional studies will be needed to clarify this point.

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In addition, the associations of IGF-I levels with SNP P0828 in *IGFALS* and P0453 in *IGFBP3* are either chance findings or real findings which bear little importance, due to the small numbers they are based upon.

Our results are in conflict with a study by Ukkola *et al.*, which found that carriers of the less frequent G variant allele of SNP rs3793344, located in a region of intron 1 of *IGFBP1* which harbors sequences affecting gene expression, had significantly lower circulating levels of IGF-I both before and after overfeeding. Subjects carrying two copies of the A allele had lower IGF-I concentrations before overfeeding, which were further decreased after overfeeding compared to subjects carrying the rarer G allele. This may indicate that the AA genotype results in higher IGFBP-1 concentrations which could decrease available IGF-I. We found no evidence of higher IGF-I concentrations with this polymorphism. It is possible that the association reported by Ukkola *et al.*, is linked to the peculiar study conditions (long-term caloric surplus). Alternatively, the association could be due to a statistical fluctuation caused by the small sample size of the study (12 pairs of monozygotic twins).

The finding supported by the strongest statistical evidence in our study is the association of polymorphisms in the 5' region of *IGFBP3* with circulating levels of IGFBP-3. This has been reported previously, and ascribed to a polymorphism located at position -202 (P0832) from the transcription start site (Deal *et al.*, 2001; Jernstrom *et al.*, 2001b; Schernhammer *et al.*, 2003). In accordance with the previous reports, we have also found a dose-dependent association of the A allele with increased levels of circulating IGFBP-3. We have found strong associations of all surrounding polymorphisms with IGFBP-3 levels (Table 3). However, an *in vitro* construct which included only SNP P0832, among the ones we have studied, found higher promoter activity of the A allele, suggesting that P0832 is the functional variant which affects IGFBP-3 transcription, and that the associations we have observed with the other polymorphisms of *IGFBP3* are due to strong LD.

The -202 polymorphism has been studied in relation to risk of breast cancer (Deal *et al.*, 2001; Schernhammer *et al.*, 2003), but without success. Likewise, we noted no association between this allele in our series as a whole or in our stratified analysis based on age of onset. Probably the effect of this polymorphism on circulating IFGBP-3 levels (estimated in 6-9% of variation Jernstrom *et al.*, 2001b; Schernhammer *et al.*, 2003) is not sufficient, by itself, to alter the amount of bioavailable IGF-I in a manner that is reflected in cancer risk.

The other variants that resulted associated with IGFBP-3 levels (a haplotype in the 5' region of IGF-I, in homozygosity, and SNP P0828 of *IGFALS*) are difficult to evaluate, due to the small numbers of subjects the associations are based upon.

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The main findings of our study were a weak but nominally significant association of a block of polymorphisms located at the 5' of the *IGF1* gene with breast cancer risk, particularly among women younger than 55, and an association of polymorphisms located in the 5' region of *IGFBP3* with circulating levels of IGFBP-3. The large numbers of statistical tests we have performed raises the issue of potential false positives. An alternative to applying a Bonferroni's correction, which is considered too conservative, is the use of a Bayesian approach, such as the recently introduced calculation of False Positive Report Probability (Wacholder *et al.*, 2004).

Given the absence of previous functional or epidemiologic data on the *IGF1* SNPs we found associated with breast cancer risk, we calculated FPRPs by using a prior probability of true association ranging of 0.01. This resulted in high FPRPs for both the overall association and the association observed among younger women. On the other hand, the association of *IGFBP3* SNPs with IGFBP-3 level is backed by previous epidemiologic and functional findings, and is supported by very low p-values in our study, which resulted in very low FPRPs over a wide range of prior probabilities of true association.

In conclusion, our results show a number of genetic variants associated with circulating hormone levels, including a convincing association of *IGFBP3* SNPs with IGFBP-3 levels. On the other hand, we have found only weak associations of genetic variants in *IGF1*, *IGFBP1*, *IGFBP3* and *IGFALS* with breast cancer risk, which will require even larger studies to be confirmed and fully evaluated.

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Table 1. PCR primers, probes and labels.

Polymorphism[a]	Polymorphism[a]	Alleles (major>minor)	Position in gene	Codon
IGF1	***IGF1***			
P0415 C/A	rs35765	C>A	Promoter region	
P0419 C/T	rs35767	C>T	Promoter region	
P0416 A/G	rs2162679	A>G	Intron 1	
P0425 T/C	rs6220	T>C	Exon 4; 3' UTR	
P0429 C/T	rs6214	C>T	Exon 4; 3' UTR	
	IGFBP1			
P0838	rs1995051	G>A	Promoter region	
P0441/839	rs1065780	G>A	Promoter region	
P0840	rs9658194	C>A	Intron 1	
P0841	rs3828998	T>C	Intron 1	
P0842	rs3793344	A>G	Intron 1	
P0444	rs4988515	C>T	Exon 4	Cys230Cys
P0445	rs4619	A>G	Exon 4	Met253Ile
IGFBP3	***IGFBP3***			
P0845 G/A	rs2132571	G>A	Promoter region	
P0844 G/A	rs2132572	G>A	Promoter region	
P0832 G/T	rs2854744	C>A	Promoter region	
P0450 G/C	rs2471551	G>C		
P0453 C/T	P0453[b]	C>T		
P0455 A/G	rs2453839	A>G		
P0448 T/C	P0448[b]	T>C		
P0846 A/T	rs6670	A>T		
IGFALS	***IGFALS***			
P0828 T/C	rs3751893	T>C	Exon 2	Asp70Asp

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P0439 C/T	rs17559	C>T	Exon 2	Tyr462Tyr
P0829 G/A	rs2230053	G>A	Exon 2	Thr522Thr

[column in red will be omitted from the final version of the manuscript. It is left temporarily for ease of consultation]

[a] Polymorphisms are identified by their dbSNP accession number. dbSNP is accessible at http://www.ncbi.nlm.nih.gov/SNP/

[b] Internal references are used for polymorphisms not present in dbSNP

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Table 2. Associations between SNPs and breast cancer risk and mean IGF-I and IGFBP-3 levels[a] adjusted for age and center

Gene	SNP		Genotype			$P_{Codominant}$[b]	$P_{Dominant}$[c]	$P_{Recessive}$[d]
			Homozygous major	Heterozygous	Homozygous minor			
IGF1	P0429 C/T	Cases/Controls	282/503	366/753	131/271			
		OR (95% CI)[e]	1.00	0.88 (0.72-1.06)	0.88 (0.68-1.13)	0.22	0.15	0.64
		Mean IGF-I[f]	245.1	243.3	244.4	0.77	0.61	0.92
		Mean IGFBP-3[f]	3556	3508	3538	0.45	0.18	0.78
	P0425 T/C	Cases/Controls	405/813	325/592	59/126			
		OR (95% CI)[e]	1.00	1.11 (0.92-1.33)	0.95 (0.68-1.32)	0.69	0.39	0.54
		Mean IGF-I[f]	240.3	248.5	240.3	0.11	**0.02**	0.51
		Mean IGFBP-3[f]	3539	3519	3511	0.46	0.46	0.71
	P0416 A/G	Cases/Controls	570/1060	212/446	19/61			
		OR (95% CI)[e]	1.00	0.88 (0.72-1.06)	0.57 (0.34-0.97)	**0.03**	0.07	0.05
		Mean IGF-I[f]	241.9	249.6	236.5	0.15	**0.04**	0.34
		Mean IGFBP-3[f]	3543	3540	3313	0.08	0.37	**0.003**
	P0419 C/T	Cases/Controls	549/1016	201/432	22/62			
		OR (95% CI)[e]	1.00	0.86 (0.71-1.05)	0.66 (0.40-1.09)	**0.04**	0.07	0.15
		Mean IGF-I[f]	242.1	251.1	236.7	0.10	**0.02**	0.30
		Mean IGFBP-3[f]	3540	3547	3364	0.23	0.63	**0.02**
	P0415 C/A	Cases/Controls	609/1148	169/362	17/37			
		OR (95% CI)[e]	1.00	0.88 (0.72-1.08)	0.86 (0.48-1.54)	0.22	0.21	0.70
		Mean IGF-I[f]	241.8	251.3	232.2	0.09	**0.02**	0.21
		Mean IGFBP-3[f]	3528	3560	3505	0.53	0.41	0.75

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Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$ [b]	$P_{Dominant}$ [c]	$P_{Recessive}$ [d]
			Homozygous major	Heterozygous	Homozygous minor			
IGFALS	P0829 G/A	Cases/Controls	788/1551	13/16	0/1			
		OR (95% CI)[e]	1.00	1.64 (0.75-0.36)	-	0.37	0.28	-
		Mean IGF-I [f]	243.6	245.5	236.6	0.92	0.90	0.92
		Mean IGFBP-3 [f]	3533	3635	3904	0.36	0.39	0.59
	P0439 C/T	Cases/Controls	620/1246	149/256	5/10			
		OR (95% CI)[e]	1.00	1.17 (0.94-1.46)	1.02 (0.35-2.99)	0.19	0.17	1.00
		Mean IGF-I [f]	243.6	246.0	226.3	0.82	0.65	0.32
		Mean IGFBP-3 [f]	3536	3497	3587	0.39	0.33	0.74
	P0828 T/C	Cases/Controls	546/1085	231/436	21/42			
		OR (95% CI)[e]	1.00	1.06 (0.88-1.28)	0.98 (0.57-1.67)	0.67	0.59	0.89
		Mean IGF-I [f]	244.7	242.5	216.7	**0.02**	0.14	**0.001**
		Mean IGFBP-3 [f]	3548	3509	3367	**0.03**	0.09	0.05

APPENDIX 5

Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$[a]	$P_{Dominant}$[b]	$P_{Recessive}$[c]
			Homozygous major	Heterozygous	Homozygous minor			
IGFBP1	rs1995051	Cases/Controls[d]	412/765	301/627	60/109			
		OR (95% CI)[e]	1.00	0.89 (0.74-1.06)	1.03 (0.74-1.44)			
		Mean IGF-I[f]	234.44	238.82	231.08	0.63	0.33	0.26
	rs1065780	Cases/Controls[d]	303/555	355/739	114/207			
		OR (95% CI)[e]	1.00	0.89 (0.73-1.07)	1.02 (0.78-1.33)			
		Mean IGF-I[f]	236.56	236.45	236.86	0.74	0.85	0.74
	rs9658194 P0840 C>A	Cases/Controls[d]	497/958	252/497	25/51			
		OR (95% CI)[e]	1.00	0.98 (0.81-1.19)	0.95 (0.59-1.55)			
		Mean IGF-I[f]	236.29	235.56	231.37	0.61	0.56	0.71
	rs3828998 P0841 T>C	Cases/Controls[d]	290/521	332/685	110/194			
		OR (95% CI)[e]	1.00	0.87 (0.72-1.06)	1.03 (0.78-1.35)			
		Mean IGF-I[f]	235.56	235.23	236.28	0.93	0.83	0.98
	rs3793344 P0842 A>G	Cases/Controls[d]	304/549	349/740	115/203			
		OR (95% CI)[e]	1.00	0.85 (0.70-1.03)	1.03 (0.79-1.35)			
		Mean IGF-I[f]	236.03	236.14	236.31	0.95	0.96	0.96
	rs4988515 P0444 C>T	Cases/Controls[d]	714/1411	62/111	2/6			
		OR (95% CI)[e]	1.00	1.11 (0.80-1.54)	0.67 (0.13-3.30)			
		Mean IGF-I[f]	235.46	244.71	230.29	0.14	0.81	0.11

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rs4619 P0445 A>G	Cases/Controls [d]	346/665	347/697	89/175			
	OR (95% CI)[e]	1.00	0.96 (0.80-1.15)	0.98 (0.73-1.31)			
	Mean IGF-I[f]	236.15	235.76	238.53	0.75	0.57	0.96

[Calculations for *IGFBP1* are being redone by Laure]

APPENDIX 5

Table 2. Cont'd

Gene	SNP		Genotype			$P_{Codominant}$ [b]	$P_{Dominant}$ [c]	$P_{Recessive}$ [d]
			Homozygous major	Heterozygous	Homozygous minor			
IGFBP3	P0846 A/T	Cases/Controls	445/887	287/572	43/61			
		OR (95% CI)[e]	1.00	1.00 (0.83-1.19)	1.44 (0.95-2.18)	0.35	0.69	0.08
		Mean IGF-I [f]	245.2	241.9	238.7	0.18	0.21	0.45
		Mean IGFBP-3 [f]	3531	3524	3618	0.58	0.92	0.19
	P0448 T/C	Cases/Controls	566/1116	202/384	14/26			
		OR (95% CI)[e]	1.00	1.05 (0.86-1.29)	1.07 (0.56-2.06)	0.61	0.61	0.87
		Mean IGF-I [f]	245.2	240.4	237.8	0.12	0.12	0.57
		Mean IGFBP-3 [f]	3522	3571	3570	0.14	0.12	0.74
	P0455 A/G	Cases/Controls	523/985	236/502	27/59			
		OR (95% CI)[e]	1.00	0.89 (0.74-1.08)	0.87 (0.55-1.39)	0.22	0.20	0.67
		Mean IGF-I [f]	244.1	243.9	245.9	0.92	0.99	0.80
		Mean IGFBP-3 [f]	3533	3522	3575	0.98	0.84	0.54
	P0453 C/T	Cases/Controls	770/1506	10/29	-			
		OR (95% CI)[e]	1.00	0.68 (0.33-1.39)	-	0.29	0.29	-
		Mean IGF-I [f]	243.8	279.8	-	**0.001**	**0.001**	-
		Mean IGFBP-3 [f]	3534	3359	-	0.12	0.12	-
	P0450 G/C	Cases/Controls	485/928	248/508	38/59			
		OR (95% CI)[e]	1.00	0.92 (0.76-1.12)	1.22 (0.80-1.86)	0.97	0.62	0.29
		Mean IGF-I [f]	242.4	243.2	247.0	0.58	0.69	0.54
		Mean IGFBP-3 [f]	3567	3470	3303	**<0.0001**	**0.0001**	**0.001**
	P0832 G/T	Cases/Controls	208/407	368/743	200/369			
		OR (95% CI)[e]	1.00	0.97 (0.79-1.20)	1.08 (0.85-1.38)	0.52	0.92	0.34
		Mean IGF-I [f]	250.2	240.2	246.5	0.32	**0.01**	0.39

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	Mean IGFBP-3 [f]	3372	3530	3677	**<0.0001**	**<0.0001**	**<0.0001**
P0844 G/A	Cases/Controls	501/980	234/478	42/59			
	OR (95% CI)[e]	1.00	0.96 (0.80-1.17)	1.42 (0.94-2.15)	0.47	0.90	0.08
	Mean IGF-I [f]	244.9	240.6	265.2	0.42	0.68	**0.002**
	Mean IGFBP-3 [f]	3566	3473	3503	**0.01**	**0.003**	0.66
P0845 G/A	Cases/Controls	393/714	308/674	75/135			
	OR (95% CI)[e]	1.00	0.83 (0.69-0.99)	1.01 (0.74-1.38)	0.29	0.08	0.50
	Mean IGF-I [f]	244.0	242.9	247.3	0.80	0.91	0.44
	Mean IFGBP-3[f]	3604	3484	3324	**<0.0001**	**<0.0001**	**<0.0001**

[a] OR = Odds ratio; CI = confidence interval ; IGF-I and IGFBP3 levels expressed in ng/mL
[b] P-value for codominant model (trend)
[c] P-value for dominant model
[d] P-value for recessive model
[e] OR
[f] Means adjusted for age and center

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Table 3a. Associations between haplotypes in *IGF1* and breast cancer risk and mean IGF-I and IGFBP-3 levels.

IGF1 Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Recessive
hCTACC	0.40	OR (95% CI)[b]	1.00	1.00	1.00
		Alpha IGF-I (*P*)[c]	418.9	417.1	425.4
		Alpha IGFBP-3 (*P*)[c]	2505	2506	2505
hTTACC	0.24	OR (95% CI)[b]	0.94 (0.79-1.12)	0.88 (0.70-1.10)	1.06 (0.74-1.52)
		Beta IGF-I (*P*)[c]	3.55 (0.22)	6.55 (0.08)	-3.40 (0.57)
		Beta IGFBP-3 (*P*)[c]	-11.36 (0.69)	-6.68 (0.86)	-26.19 (0.66)
hCCACC	0.10	OR (95% CI)[b]	1.07 (0.83-1.38)	1.03 (0.77-1.39)	1.59 (0.72-3.49)
		Beta IGF-I (*P*)[c]	8.06 (0.06)	9.65 (0.05)	12.88 (0.35)
		Beta IGFBP-3 (*P*)[c]	-14.32 (0.73)	-21.46 (0.66)	136.48 (0.32)
hTCACC	0.08	OR (95% CI)[b]	1.07 (0.82-1.39)	1.05 (0.78-1.42)	1.63 (0.68-3.94)
		Beta IGF-I (*P*)[c]	-3.03 (0.48)	-2.58 (0.59)	-23.80 (0.12)
		Beta IGFBP-3 (*P*)[c]	7.90 (0.85)	-5.01 (0.92)	261.32 (0.11)
hCCGTA	0.06	OR (95% CI)[b]	0.86 (0.65-1.14)	0.84 (0.62-1.14)	0.80 (0.25-2.56)
		Beta IGF-I (*P*)[c]	**9.25 (0.04)**	**12.56 (0.01)**	-20.36 (0.28)
		Beta IGFBP-3 (*P*)[c]	-20.56 (0.64)	-22.78 (0.64)	20.35 (0.91)
hTTGTA	0.05	OR (95% CI)[B]	0.89 (0.65-1.22)	0.89 (0.63-1.24)	0.75 (0.20-2.85)
		Beta IGF-I (*P*)[c]	1.76 (0.72)	3.12 (0.56)	-17.95 (0.39)
		Beta IGFBP-3 (*P*)[c]	33.50 (0.50)	41.09 (0.44)	-25.92 (0.90)
hTCGTC	0.02	OR (95% CI)[B]	0.79 (0.48-1.32)	0.79 (0.47-1.31)	-
		Beta IGF-I (*P*)[c]	4.77 (0.56)	5.16 (0.53)	-
		Beta IGFBP-3 (*P*)[c]	**-179.43 (0.03)**	**-181.79 (0.02)**	-
hTTGTC	0.01	OR (95% CI)[B]	**0.40 (0.20-0.81)**	**0.40 (0.20-0.81)**	-
		Beta IGF-I (*P*)[c]	-3.17 (0.75)	-2.65 (0.79)	-
		Beta IGFBP-3 (*P*)[c]	**-285.84 (0.003)**	**-250.87 (0.01)**	-
hCTGTA	0.01	OR (95% CI)[B]	0.80 (0.35-1.85)	0.77 (0.33-1.78)	-
		Beta IGF-I (*P*)[c]	11.52 (0.38)	12.23 (0.36)	-
		Beta IGFBP-3 (*P*)[c]	-14.03 (0.91)	-16.09 (0.90)	-

[a] OR = Odds ratio; CI = confidence interval

[b] Reference group = hCTACC

[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center

[d] The order of SNPs in the haplotype corresponds to their physical order: P0429, P0425, P0416, P0419, P0415.

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Table 3b. Associations between haplotypes in *IGFBP1* and breast cancer risk and mean IGF-I and IGFBP-3 levels.

[a] The order of SNPs in the haplotype corresponds to their physical order: rs1995051, rs1065780, rs9658194, rs3828998, rs3793344, rs4988515, rs4619
[b] OR = Odds ratio; CI = confidence interval; subjects are matched for age
[f] Beta estimate for IGF-I levels in ng/ml for a one unit change in haplotype dosage adjusted for age and center

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Table 3c. Associations between haplotypes in *IGFBP3* and breast cancer risk and mean IGF-I and IGFBP-3 levels.

IGFBP-3 Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Recessive
hATACGGAG	0.18	OR (95% CI)[b]	1.00	1.00	1.00
		Alpha IGF-I (*P*)[c]	424.6	427.6	424.1
		Alpha IGFBP-3 (*P*)[c]	2454	2487	2509
hATACGTGG	0.17	OR (95% CI)[b]	0.99 (0.79-1.23)	0.92 (0.72-1.18)	1.15 (0.71-1.86)
		Beta IGF-I (*P*)[c]	2.80 (0.44)	-0.04 (0.99)	**18.74 (0.02)**
		Beta IGFBP-3 (*P*)[c]	**136.49 (0.0001)**	**122.82 (0.002)**	**243.49 (0.003)**
hACACGTGG	0.12	OR (95% CI)[b]	1.06 (0.83-1.27)	1.04 (0.80-1.35)	1.02 (0.45-2.30)
		Beta IGF-I (*P*)[c]	-5.10 (0.21)	-7.67 (0.07)	0.66 (0.96)
		Beta IGFBP-3 (*P*)[c]	**107.69 (0.007)**	**95.50 (0.02)**	103.18 (0.42)
hTTACCGGA	0.09	OR (95% CI)[b]	0.95 (0.72-1.24)	0.84 (0.63-1.12)	**2.66 (1.10-6.42)**
		Beta IGF-I (*P*)[c]	-1.16 (0.79)	-4.52 (0.32)	24.23 (0.11)
		Beta IGFBP-3 (*P*)[c]	-67.12 (0.12)	**-103.64 (0.02)**	-68.24 (0.65)
hTTACGTGG	0.09	OR (95% CI)[b]	1.08 (0.81-1.42)	1.02 (0.76-1.37)	1.86 (0.72-4.83)
		Beta IGF-I (*P*)[c]	-5.36 (0.24)	-7.59 (0.11)	2.11 (0.90)
		Beta IGFBP-3 (*P*)[c]	**159.65 (0.0004)**	**146.59 (0.002)**	276.50 (0.09)
hATGCGGGA	0.09	OR (95% CI)[B]	0.92 (0.70-1.21)	0.85 (0.64-1.12)	2.00 (0.64-6.26)
		Beta IGF-I (*P*)[c]	-2.99 (0.49)	-4.36 (0.32)	-6.81 (0.74)
		Beta IGFBP-3 (*P*)[c]	-28.57 (0.50)	-51.54 (0.24)	-124.30 (0.54)
hATACCGGA	0.08	OR (95% CI)[B]	1.01 (0.76-1.34)	0.93 (0.69-1.27)	1.71 (0.66-4.44)
		Beta IGF-I (*P*)[c]	-0.60 (0.90)	-1.70 (0.74)	-2.12 (0.90)
		Beta IGFBP-3 (*P*)[c]	1.24 (0.98)	-23.88 (0.63)	-27.24 (0.87)
hATGCGTGG	0.07	OR (95% CI)[B]	0.90 (0.66-1.23)	0.83 (0.60-1.15)	1.45 (0.39-5.46)
		Beta IGF-I (*P*)[c]	4.36 (0.39)	3.65 (0.49)	-24.55 (0.29)
		Beta IGFBP-3 (*P*)[c]	**104.84 (0.04)**	98.41 (0.06)	-162.52 (0.48)
hTTGCGTGG	0.02	OR (95% CI)[B]	0.68 (0.38-1.22)	0.66 (0.37-1.19)	-
		Beta IGF-I (*P*)[c]	-10.93 (0.24)	-12.59 (0.17)	-
		Beta IGFBP-3 (*P*)[c]	**226.81 (0.01)**	**203.94 (0.03)**	-
hATACGGGA	0.02	OR (95% CI)[B]	**0.52 (0.29-0.95)**	**0.51 (0.27-0.94)**	-
		Beta IGF-I (*P*)[c]	-9.72 (0.25)	-13.75 (0.12)	-
		Beta IGFBP-3 (*P*)[c]	-126.80 (0.13)	-127.39 (0.15)	-

[a] OR = Odds ratio; CI = confidence interval

[b] Reference group = hATACGGAG

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[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center
[d] The order of SNPs in the haplotype corresponds to their physical order: P0846, P0448, P0455, P0453, P0450, P0832, P0844, P0845.

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Table 3d. Associations between haplotypes in *IGFALS* and breast cancer risk and mean IGF-I and IGFBP-3 levels.

IGFALS Haplotypes [d]	Haplotype Frequency		Model		
			Codominant	Dominant	Recessive
hGTC	0.73	OR (95% CI)[b]	1.00	1.00	1.00
		Alpha IGF-I (*P*)[c]	427.5	426.0	428.0
		Alpha IGFBP-3 (*P*)[c]	2551	2542	2531
hGCC	0.17	OR (95% CI)[b]	1.06 (0.90-1.25)	1.08 (0.89-1.29)	0.98 (0.58-1.67)
		Beta IGF-I (*P*)[c]	**-6.21 (0.02)**	-4.42 (0.15)	**-27.93 (0.001)**
		Beta IGFBP-3 (*P*)[c]	**-59.79 (0.03)**	-55.27 (0.07)	**-173.22 (0.04)**
hGTT	0.10	OR (95% CI)[b]	1.15 (0.93-1.41)	1.16 (0.93-1.44)	1.14 (0.41-3.14)
		Beta IGF-I (*P*)[c]	-0.37 (0.92)	0.97 (0.79)	-19.15 (0.28)
		Beta IGFBP-3 (*P*)[c]	-39.61 (0.25)	-43.09 (0.24)	51.87 (0.77)

[a] OR = Odds ratio; CI = confidence interval

[b] Reference group = hGCT

[c] Beta estimate for IGF-I and IGFBP-3 levels (in ng/mL) for a one unit change in haplotype dosage adjusted for age and center

[d] The order of SNPs in the haplotype corresponds to their physical order: P0829, P0439, P0828.

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Supplementary Table 1. PCR primers, probes and labels.

SNP	Polymorphism[a]	Primers (5'-3')	Probes (5'-3')[b]
	IGF1		
P0415		CATCTTCATACAATATTAATGACACA TCCAATTCTAAGTTTTCTTACTCATT	HEX-LNA-CTT+CTCT+TTGG+CAA+TGATC FAM-LNA- CTT+CTCTTT+GG+AA+AT+GATC
P0419		ABI assay-on-demand C____799146_10[c]	ABI assay-on-demand C____799146_10[c]
P0416		TCTTCCAGTCCCAGTGTTTTGG GAGGAGGTAAAAGCTCAACTGAACA	VIC-MGB-ATCTTCCACCCTCTACATAG FAM-MGB-TTCCACCCCCTACATAG
P0425		AACAAAGAGATTTCTACCAGTGAAAGG GCCTAGAAAAGAAGGAATCATTGTG	VIC-MGB-AGTAAAACCTCGTTTAATA FAM-MGB-AGTAAAACCTTGTTTAATAC
P0429		TCTCAACAAAACTTTATAGGCAGTCTTC GTGAAGGAAATAAGTCATAGACACTCTTAGAA	VIC-MGB-TGCAGACTTAACATGTT FAM-MGB-AGTCATGCGGAAAA
	IGFBP1		
P0838	rs1995051	GTTCCCAGAAATGGCTGTGTGA GTCCATCCGTCCATCCTGTGT	HEX-MGB- CACATCATCGGCTGTGACTCCAACCTGC FAM-MGB- CACATCATCGGCTGTAACTCCAACCTGC
P0441/839	rs1065780	CCCCATCTCGCCTTTCCT GCAAAAATTTAAAAATCCTGGGTAAC	VIC-MGB-TCTCAAATGCAGAAAA FAM-MGB-CGTGAGAGGATTGAG
P0840	rs9658194	GGGAAGGAGCTTGGGTCACC TCCTTGAGTCTCCACTAAGCTATGT	HEX- AGCCCGCTCATTGAACGGTCTTGGCA FAM- AGCCCGCTCATTGCACGGTCTTGGCA
P0841	rs3828998	CCCGCTCATTGCACGGTCTT GTCTCCACTAAGCTATGTGTGCC	HEX-LNA-CTCC+CAG+AG+CACGTC FAM-LNA-CCC+AG+GGC+ACGT
P0842	rs3793344	TGGAGACTCAAGGAGGAAGCTC GTGCTGGCAAGGAGACTGGT	HEX- CCTCGTAGCCCAGGGATCTTTAGAGACCC FAM- CCTCGTAGCCCAGAGATCTTTAGAGACCC
P0444	rs4988515	GTCTTTGCAGTGTGAGACATCCAT GGAGACCCAGGGATCCTCTTC	VIC-MGB-TGGTGCGTCTACC FAM-MGB-TGGTGTGTCTACCC
P0445	rs4619	CCCTGGGTCTCCAGAGATCAG TTTCACGTGACAGAACATTATTTCATC	VIC-MGB-TGCCAGATGTATTTTA FAM-MGB-CTGCCAGATATATTT
	IGFBP3		
P0845		GTTAATTACGTTTCAGCAGTGC TTAAGGCAGGGCTTTTCAAAT	HEX-LNA-TTT+TGTC+GTGGG+TG+TAA FAM-LNA- TCT+TT+TGTC+ATGGG+TG+TAA
P0844		TTAAGGACGCATTCGCTTGC GCCCACAAATACGCATCTGA	HEX-LNA- CTTAAT+TGG+GGACT+TGCG FAM-LNA- CTT+AAT+TGG+AGA+CTTGCGG
P0832		CACCTTGGTTCTTGTAGACGACAA GGCGTGCAGCTCGAGACT	VIC-MGB-TCCTCGTGCGCACG FAM-MGB-CTCGTGCTCACGCC

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P0450	ACCTGGTTGCAACGTTAAGATTTT	VIC-MGB-TTGTCTCTCTTGGGCG
	CCGACTCACTAGCATTTCCTTAAAA	FAM-MGB-TGTCTGTCTTGGGCG
P0453	CTGCTGGTGTGTGGATAAGTATGG	GTAGCAGTGCACGTCCTCCTT
	GTAGCAGTGCACGTCCTCCTT	FAM-MGB-CCCTTGATGGTGTAGC
P0455	GCATCTGGTACTCGTTGCTTTGT	VIC-MGB-CTCAACTCATGTTTTC
	CCTGCAGGCTAATGGCACTAG	FAM-MGB-CAACTCACGTTTTCA
P0448	GGCCCAGGATGGCTTTTG	VIC-MGB-AGAGACAGGGAGAGTC
	ATTACTTGTGATGCCTCTGAATGTG	FAM-MGB-AGAGACGGGAGAGTC
P0846	CTATACTAGATAATCCTAGATGAAATGT	HEX-LNA-ATG+CTAT+TT+GATA+CAA+CTGT
	GCCAGACCTTCTTGGGTT	FAM-LNA-TGC+TAT+AT+GATA+CAA+CTGT
	IGFALS	
P0828	CTGCAGCTCCAGGAACCT	VIC-MGB-CCCGGGACTCCGTCAG
	GCCACAGGGCTTGGGT	FAM-MGB-CCGGGACTCCATCAG
P0439	CTCCAACCAGCTCACGC	HEX-LNA-AAGC+TG+GAG+TA+TC+TGC
	GAGACGTCCAGCCAGAA	FAM-LNA-AAGC+TGG+AGTA+CC+TGC
P0829	CAGCCTCAGGAACAACTCACT	VIC-MGB-CCTTCACACCGCAGCC
	GCCACAGGCGCTCCA	FAM-MGB-TCACGCCGCAGCC

(column in red will be omitted from the final version of the manuscript. It is left temporarily for ease of consultation)

[a] Polymorphisms are identified by their dbSNP accession number. dbSNP is accessible at http://www.ncbi.nlm.nih.gov/SNP/

[b] Fluorescent dye and presence of stabilizing molecules (MGB or LNA) are indicated. For LNA probes, a plus sign indicates bases modified with an LNA molecule

[c] No information on sequences of primers and probes is available for assays purchased as predesigned Assays-on-demand from Applied Biosystems

APPENDIX 5

Figure Legends

Figure 1a. Graphical representation of LD and block structure of *IGF1*.

Figure 1b. Graphical representation of LD and block structure of *IGFBP3*.

The upper bars represent SNPs and physical distances among them. Numbers within squares are pairwise D' values. Absence of value means D'=1. The color code shows confidence boundaries of LD estimations: black shows evidence of LD, white shows evidence of recombination, gray shows uninformative pairs. LD blocks were defined accoridng to the algorithm of Gabriel *et al.* (Gabriel *et al.*, 2002)

APPENDIX 5

Figure 1a

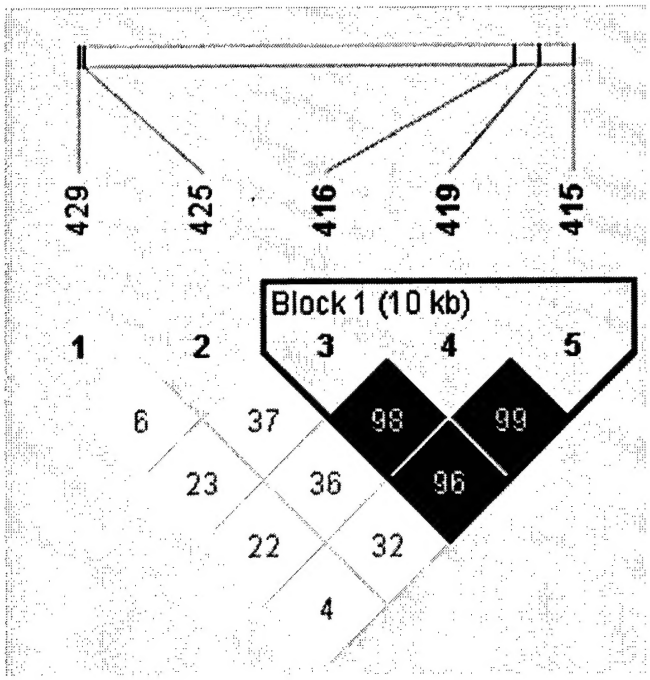
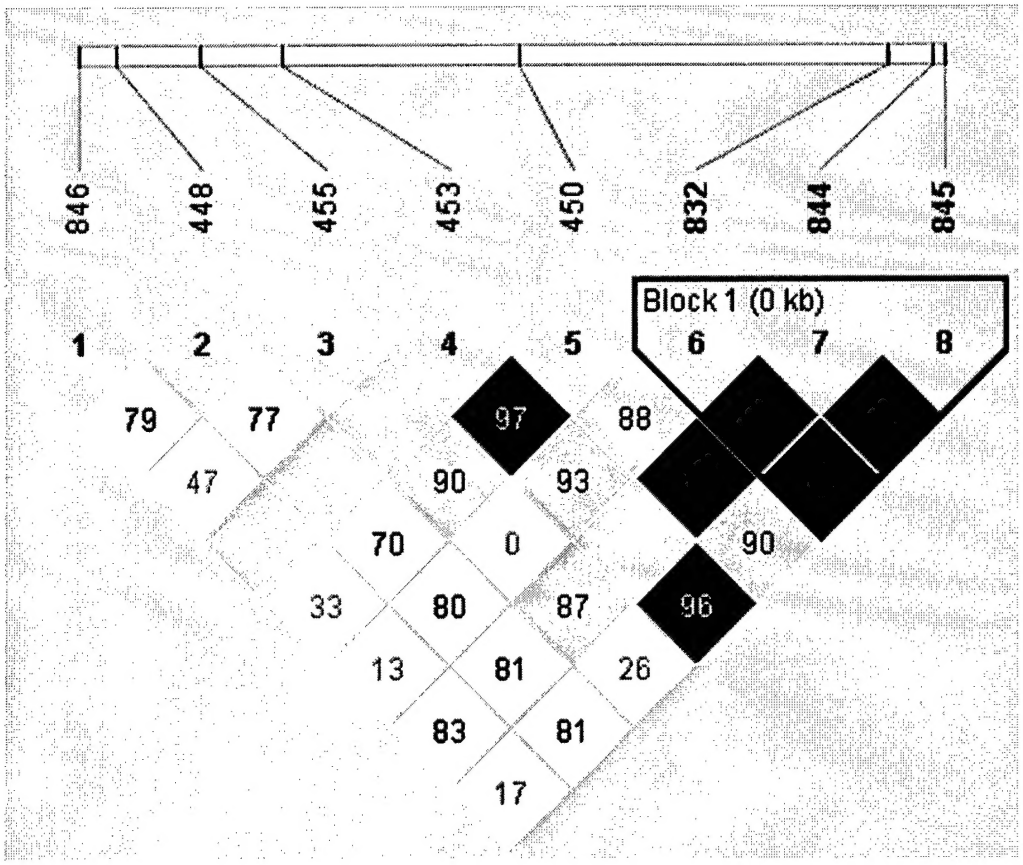


Figure 1b



APPENDIX 6

GENETIC DETERMINANTS OF PLASMA IGF-I LEVELS

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Insulin-like growth factor-I (IGF-I) can enhance the development of tumors in different organs, including the breast. It is therefore important to understand what factors can lead to elevated IGF-I in the circulation and tissues. One well-documented determinant of circulating IGF-I levels is nutritional status, especially the availability of energy and essential amino acids from diet and body reserves. However, heritability studies comparing homozygous and heterozygous twins have shown that, in western populations, 40-60 % of variation in IGF-I is (co-)determined by genetic factors.

We have started a cross-sectional study to examine relationships of single-nucleotide polymorphisms (SNPs) in 14 candidate genes in the growth-hormone (GH) / IGF-I synthesis pathway. An exhaustive catalog of polymorphisms (coding and non-coding regions), was made by searching MEDLINE and genomic databases, and by experimental discovery using partially denaturing HPLC (DHPLC) in DNA samples from 137 Caucasian, 43 African, and 12 Japanese subjects. For 327 healthy men and women, aged 50 - 60, living in Umeå, Northern Sweden, these SNPs were typed using hybridization coupled with an enzyme-mediated primer extension on a DNA microarray. Of 60 SNPs typed successfully, 51 had a population prevalence above 1%, and no strong linkage disequilibrium with other SNPs (Δ<0.90). For about 80% of individuals who were homozygous for all, or for all but one of the SNPs, SNP haplotypes could be calculated; for the remaining subjects haplotypes were estimated from population haplotype frequencies.

Gene	Number of different alleles		nr of SNP loci showing effects / model p-value / model R^2, for selected regression models
	SNPs	Haplotypes	
IGF1	4	6	2 / p = 0.10 / R^2 = 0.03
IGFBP3	5	13	3 / p = 0.03 / R^2 = 0.04
GH1	3	4	no significant effects
GHR	5	7	1 / p = 0.06 / R^2 = 0.02
GHRH	2	3	1 / p = 0.06 / R^2 = 0.02
GHRHR	10	15	
SST	1		no significant effects
SSTR1	1		no significant effects
SSTR3	6	12	3 / p = 0.06 / R^2 = 0.05
SSTR4	4	9	no significant effects
SSTR5	4	9	no significant effects
POU1F1	1		no significant effects
GHRL	4	9	2 / p = 0.08 / R^2 = 0.04
GHSR	1		1 / p = 0.02 / R^2 = 0.02

Statistical models for phased SNP genotypes, selected by forward selection [p_{IN}< 0.15] and backward elimination [$p_{OUT} \geq 0.15$], showed evidence that polymorphic variation in the candidate genes may alter plasma IGF-I levels, although associations were only of borderline statistical significance, and the percent variation in IGF-I levels explained by individual gene variants was small. After increasing the sample size of the study, we plan to estimate a combined score for multiple candidate genes.

We expect this project to increase understanding of how cancer risk (breast and other organs) may be determined by dysregulations in the GH/IGF-I axis, and how such dysregulations may be codetermined by specific genetic susceptibility factors.

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APPENDIX 7

DAMD17-01-1-0275
Breast cancer risk in relation to serum IGF-I, IGFBP-3 and their genetic determinants: A study within the European Prospective Investigation into Cancer (EPIC)

A list of personnel (not salaries) receiving pay from the research effort

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Pietro Ferrari
Wira Fevre-Hlaholuk
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